

The Generation and Characterisation of Anti-Glycan Monoclonal Antibodies

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, July 2016

I dedicate this thesis to my father (Guan Bok) and my mother (Guik Yen)

Acknowledgement

First and foremost I want to thank my supervisor Professor Lindy Durrant. It has been an honour to be her Ph.D. student. I appreciate all her contributions of time, ideas and funding to make my Ph.D. experience productive and stimulating. The joy and enthusiasm she has for her research was motivational for me, even during tough times in the Ph.D. pursuit. I am also thankful for the excellent example she has provided as a successful scientist.

I would also like to thank my co-supervisor, Associate Professor Dr. Ian Spendlove and my internal assessor, Dr. Judith Ramage for being very supportive and providing me help throughout my Ph.D.

The members of the antibody group have contributed immensely to my personal and professional time at the University of Nottingham. The group has been a source of friendship as well as good advice and collaboration. I am especially grateful for my dream team member, Robert Moss. We worked together to generate therapeutic monoclonal antibodies, and I very much appreciated his enthusiasm, intensity, amazing ability in the laboratory and most importantly, his willingness in sharing his knowledge. Other past and present group members that I have had pleasure to work with or alongside are postdocs Dr. Richard McIntosh, Dr. Mireille Vankemmelbeke, Dr. Tina Parsons, Dr. Catherine Rabu, Dr. Ihsun Chiang, Dr. Zuzana Jurasova and Dr. Philip Noble; Ph.D. student Nur Adilah and the numerous master and BMed science students who have come through the laboratory.

In my latter work of studying monoclonal antibody cytotoxicities using scanning electron microscopy and confocal microscopy, I would like to thank Gray Trevor, Christie Denise and Tim Self for inspirational and helpful discussions. I also thank Phil Clarke for carrying out the animal studies. Special thanks go to Dr Emili Cid and Dr Fumiichiro Yamamoto (IJC Josep Carreras Leukemia Research Institute, Barcelona) for providing engineered cell lines.

For this Ph.D. thesis I would like to thank my reading committee members Dr. Mireille Vankemmelbeke and Dr. Tina Parsons for their time, interest and helpful comments.

I gratefully acknowledge the funding source that made my Ph.D. work possible. I was funded by the University of Nottingham Vice Chancellor Research Excellence scholarship for international student for my first three years and was honoured to be a graduate fellow granted by Bowel Cancer Trust.

Special thanks go to Christopher Nolan and Mohamed Khaled Gijon (sun wu kong) for giving me their time and space throughout my Ph.D. ‘I just wanted to say thank you and let you know how grateful I am for all the help and support that both of you have given me over the past 7 years. As you are

aware, this year has been one of the hardest for me and I don't think that I would still be here today if it wasn't for all the support I've received from both of you'.

My time in the United Kingdom was made enjoyable in large part due to the many friends and groups that became a part of my life. I am grateful for time spent with Melissa Ooi, Duobui Chua, Yi Lin Lee, Eugenia Lim, Jason Lim, Chia Wei Phan, Lorinda Liew, Sarene Saw, Wei Fen Hue, Jacqueline Ting, Phey Feng Lo, Cecilia Tang, Qing Xi Ooi, Li Mei Lim, Josephine Song, Mohamed Khaled Gijon, Christopher Nolan, Ihsun Chiang, Giota Chondrou (100KM), Veronica Martini, Ivy Tang, Poonam Bilimoria (ice cream), Arancha Diez, Zuzana Jurasova, Nur Adilah, Qun Wei Wang, Yi Min Zhang, Nurul Akmaryanti, An Qi Yao and Bhudsaban Sukkarn.

Lastly, I would like to thank my family for all their love and encouragement. For my parents who have raised me and supported me in all my pursuits. I also thank my best friend Jacelyn Ang for being very supportive. And most of all for my loving, supportive and encouraging family members; Peng Teng, Jia Mei, Jia Yi, Peng Jun, Wan Yun, DuoBui, Sheue Fen, Sheue Li and makmak whose faithful supports during the final stages of this Ph.D. are so appreciated. Thank you.

Jia Xin Chua

University of Nottingham

Academic Clinical Oncology

July 2016

General abbreviations

A

Activation-induced deaminase (AID)

Acute lymphoblastic leukemia (ALL)

Acute myelogeneous leukemia (AML)

Alpha-galactosylceramide (α-GalCer)

Anaplastic large cell lymphoma (ALCL)

Antibody dependent cellular cytotoxicity (ADCC)

Antibody dependent cellular phagocytosis (ADCP)

Antibody-drug conjugates (ADCs)

Antigen (Ag)

Antigen presenting cells (APCs)

Asparagine (Asn)

B

B cell receptors (BCRs)

Basal like (BL) breast cancer

Basal like triple negative breast cancer (BL-TNBC)

Beta-(1,4)-N-acetylglucosaminyltransferase III (GnTIII)

Beta-2-microglobulin (β_2m)

Bioluminescent tumour burden (BLI)

Bone marrow (BM)

Bovine serum albumin (BSA)

Breast cancer stem cell (BCSC)

C

Cancer Research UK (CRUK)

Cancer stem cells (CSCs)

Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-FMK-VAD)

Chinese hamster ovary (CHO)

Class switch recombination (CSR)

Clear cell renal cell carcinoma (ccRCC)

Coding joints (CJs)

Colorectal carcinoma (CRC)

Complement-dependent cellular cytotoxicity (CDC)

Complementarity determining regions (CDRs)

Consortium for Functional Glycomics (CFGs)

Cross reactive material (CRM)

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4)

D

3, 3'-Diaminobenzidine tetra hydrochloride (DAB)

Dendritic cells (DCs)

Decay-accelerating factor (DAF)

Dicetylphosphate (DCP)

Dithiothreitol (DTT)

Diversity (D) gene segments

Dulbecco's Modified Eagle's medium (DMEM)

DNA ligase 4 (Lig 4)

Double-strand breaks (DSBs)

E

Eagle's Minimum Essential Medium (EMEM)

Enhancer (E)

Epidermal growth factor receptor (EGFR)

Epidermal growth receptor (HER2)

Estrogen receptor (ER)

F

Fab secondary conjugated (Fab-ZAP)

Factor-I (fI)

Fetal bovine serum (FBS)

Fc receptor (FcR)

Fc-gamma receptors (FcγRs)

Fibroblast growth factor receptor (FGFR)

Follicular (FO)

Follicular dendritic cells (FDCs)

Food and Drug Administration (FDA)

Fragment antigen binding (Fabs)

Fucosyltransferase 8 (FUT8)

G

Gastrointestinal (GI)

Geometric mean (Gm)

Germinal centre (GC)

Glucosylceramide (GlcCer)

Glycosaminoglycans (GAGs)

Glycosylphosphatidylinositol (GPI)

Glycosphingolipids (GSLs)

H

Heavy (H) chain

Heat shock protein (HSP)

Heavy-chain variable (vH)

Heavy-chain constant (cH)

Hodgkin's lymphoma (HL)

Hormone receptor (HR)

Human serum albumin (HSA)

Hypoxanthine-aminopterin-thymidine (HAT)

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)

Hypoxanthine thymidine (HT)

I

IgG (γ)

IgM (μ)

IgA (α)

IgD (δ)

IgE (ε)

IL-1 receptor associated kinase (IRAK)

Immune stimulating complexes (ISCOMs)

Immunoglobulin (Ig)

Immunoglobulin heavy (IgH)

Immunoglobulin light (IgL)

Immunohistochemistry (IHC)

Immunoreceptor tyrosine activate motifs (ITAMs)

Immunoreceptor tyrosine-based inhibition motifs (ITIMs)

Innate-like B cells (ILBs)

Innate regulatory B cells (innate Bregs)

Insulin receptor (IR)

Intraperitonealy (i.p.)

Intraveneously (i.v.)

Invariant natural killer T (iNKT)

Interferon-gamma (IFN-γ)

Inducible co-stimulator (ICOS)

Insulin-like growth factor receptor (IGFR)

J

Joining (J) gene segments

K

Kilo Dalton (kDa)

Keyhole limpet haemocyanin (KLH)

L

Lactosylceramide (LacCer)

Lipopolysaccharides (LPS)

Light (L) chain

Light chain constant (cL)

Light-chain variable (vL)

Lymph node (LN)

M

Mannose binding lectin (MBL)

Marginal zones (MZ)

Major histocompatibility complex (MHC)

Membrane attack complex (MAC)

Membrane cofactor protein (MCP)

Membrane complement regulatory proteins (mCRPs)

Metastatic Pancreatic Adenocarcinoma Clinical Trial (MPACT)

Mitogen activated protein (MAP)

Monoclonal antibodies (mAbs)

N

N-hydroxysuccinimide (NHS)

Natural killer (NK) cells

Neurotrophic tyrosine kinase receptor type 1 (TrkA)

New born calf serum (NBCS)

Non-Hodgkin's lymphoma (NHL)

Non-homologous end joining (NHEJ)

Non-small cell lung cancer (NSCLC)

Normal mouse serum (NMS)

Normal swine serum (NSS)

Nottingham Prognostic Index (NPI)

O

Optical density (O.D.)

P

Pathogen-associated molecular patterns (PAMPs)

Peripheral blood (PB)

Phosphotidylcholine (PC)

Phosphatidylinositol 3-kinase (PI3K)

Polyethylene glycol (PEG)

Polymorphonuclear neutrophils (PMNs)

Progesterone receptor (PgR)

Programmed cell death protein-1 (PD-1)

Promoter (p)

R

Recombinase activating gene (RAG)

Recombination signal sequences (RSSs)

Region of Interest (ROI)

Regulatory T cells (Tregs)

Renal cell carcinoma (RCC)

S

Saporin-conjugated anti-mouse/anti-human IgG (ZAP)

Serine (Ser)

Short tandem repeat (STR)

Single stranded breaks (SSBs)

Single chain fragment variable (scFv)

Single-stranded RNA (ssRNA)

Sialyltransferases (ST)

Sialic acid-binding immunoglobulin-like lectins (Siglecs)

Somatic hypermutation (SHM)

Small cell lung cancer (SCLC)

Streptavidin Horseradish Preoxidase (HRPO)

Subcutaneously (s.c.)

Surface Plasmon Resonance (SPR)

T

[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (WST8)

3,3',5,5'-Tetramethybenzidine (TMB)

T-dependent (T-D)

T-independent (T-I)

T cell receptors (TCRs)

Terminal-deoxynucleotidyl-transferase (TdT)

Tetanus toxoid (TT)

Tissue microarrays (TMAs)

TNF receptor associated factor-6 (TRAF-6)

TNF-related apoptosis-inducing ligand receptor (TRAIL)-R

Toll-like receptor (TLR)

Toll/IL-1 receptor (TIR)

Transitional (TR)

Transforming growth factor-α (TGF-α)

Triple negative breast cancer (TNBC)

Tryptophan (Trp)

Tumour associated carbohydrate antigens (TACAs)

Tumour necrosis factor (TNFα)

Tumour necrosis factor receptor-1 (TNFR1)

Tumour propagating cells (TPCs)

V

Variable (V) gene segment

Variable heavy domain of humans (dAbs)

Vascular endothelial growth factor (VEGF)

Z

Zwitterionic polysaccharides (ZPSs)

Glycan abbreviations

Fucose (Fuc)

Galactose (Gal)

Glucose (Glc)

Lewis a/c/x ($Le^{a/c/x}$)

Lewis carbohydrate antigen (Le)

Mannose (Man)

Mucin (MUC)

N-acetylgalactosamine (GalNac)

N-acetylglucosamine (GlcNac)

Sialic acid (Sia)

Sialyl-Lewis a (SLe^{a})

Sialyl-Lewis x (SLe^{x})

Stage specific embryonic antigen-1 (SSEA-1)

Stage specific embryonic antigen-3 (SSEA-3)

Stage specific embryonic antigen-4 (SSEA-4)

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Abstract

Glycomic profiling of tumour tissues consistently shows alterations in N- and O-glycosylation profiles of glycoproteins and glycolipids compared to healthy tissues, with important functional implications for cancer cell biology. Despite the attractiveness of the targets, there are very few mAbs recognising glycans as they induce low affinity IgM responses.

The aim of this study was to develop a novel immunisation protocol, with the objective of increasing the immunogenicity of tumour-associated carbohydrate or glycolipid antigens, leading to the generation of novel anti-glycan monoclonal antibodies (mAbs) with potent cytotoxicity against cancers.

Mice were immunised with, tumour cell plasma membrane glycolipid extracts incorporated into liposomes in the presence or absence of alpha-galactosylceramide (α-GalCer) and/or anti-CD40 mAb and/or with engineered cells, which express specific glycolipids. Three IgG3 murine mAbs (FG88.2, FG88.7 and FG2811.72) with subnanomolar potency were generated, suggesting that the immunisation protocols succeeded in increasing the immunogenicity of glycolipid antigens which promoted antibody class switching and affinity maturation. FG88.2 and FG88.7 mAbs recognised the novel Lewis a/c/x ($Le^{a/c/x}$) glycans expressed on glycolipids and glycoproteins whereas FG2811.72 is an ultraspecific mAb targeting SSEA-4 glycolipid.

Immunohistochemical analysis showed that FG88.2 glyco-epitopes were overexpressed on a broad range of tumour types with limited cross reactivity with normal human tissues. Characterisation of effector functions of FG88 and FG2811.72 mAbs demonstrated that they were capable of inducing potent antibody dependent cellular cytotoxicity (ADCC; EC_{50} FG88 mAbs: 10^{-9} M; EC_{50} FG2811.72 mAb: 10^{-10} M) and complement dependent cytotoxicity (CDC; EC_{50} FG88 mAbs: 10^{-9} M; EC_{50} FG2811.72 mAb: 10^{-9} M). In addition, FG88 mAbs were capable of inducing direct tumour cell death via oncosis which induces pore formation and lysis of tumour cells. Preliminary results showed that FG2811.72 mAb inhibited tumour cell growth in the absence of immune cells and complement proteins, but the mechanism of tumour cell growth inhibition remains unexplored. The excellent *in vitro* cytotoxicity of FG88 mAbs translated into potent anti-tumour efficacy and significant survival improvement in a colorectal hepatic metastasis xenograft model in which mAb treatment was initiated 10 days after tumour initiation and development of liver metastasis. No other studies, to date, have shown tumour eradication of 10-day established tumours and liver metastases.

Traditional cancer chemotherapy is often accompanied with severe side effects to cancer patients. Antibody-drug conjugates (ADCs) are composed of a therapeutic mAb chemically linked to a potent cytotoxic drug. By linking mAbs to cytotoxic drugs, ADCs selectively target tumour cells, thus improving the therapeutic index of chemotherapeutic agents. ADCs work via the internalisation of antibody-antigen complexes into cancer cells, followed by the release of the cytotoxic drugs in the lysosomes which then causes cancer cell death. FG88 and FG2811.72 mAbs deliver Zaporin conjugates into tumour cells with subnanomolar potency, suggesting they have the potential to be used as ADCs.

Murine mAbs have limited clinical utility due to their short half-life in serum, their suboptimal in stimulation of Fc effector functions and their high immunogenicity in humans. Therefore chimeric FG88.2 (CH88.2; IgG1) mAb was generated. Although CH88.2 mAb maintained antigen specificity and affinity, it had reduced CDC activity and importantly, it lost its ability to induce direct tumour cell death. In this study, the E345R mutation [a single amino acid substitution at position 345 ($Glu^{345}\rightarrow$ Arg)] was introduced into CH88.2 to generate a mutant version of CH88.2 named ECH88.2 mAb. The ECH88.2 mAb showed restored direct cell killing ability and the CDC activity was improved. In addition, ECH88.2 demonstrated excellent ADCC activity.

Lewis a/c/x ($Le^{a/c/x}$) glycans were expressed by both glycolipids and glycoproteins. Studies on the role of glycolipids/glycoproteins in inducing direct tumour cell death suggested that it was primarily mediated by glycolipids. In contrast, both glycolipids and glycoproteins were involved in ADCC, CDC and mAb internalisation into tumour cells.

In summary, the aim of this study was achieved by generating three novel anti-glycan mAbs which bind to tumour associated carbohydrate antigens with high affinity. Their strong tumour specificity and limited cross reactivity to normal tissues, as well as their potent cytotoxicity effects, suggest that they should have great potential as therapeutic anti-cancer mAbs. Furthermore, their ability to internalise into tumour cells suggests that they may have potential as drug carriers. Importantly, this study demonstrates a novel approach: by introducing the E345R mutation in the Fc region of the chimeric mAbs it promotes non-covalent interaction in the presence of antigen leading to increased cell surface binding, improved CDC activity and restoration of the direct killing ability of the mouse mAbs.

Chapter 1: Introduction

MAbs have emerged as one of the leading players in the fight against cancer. This thesis is focused on the production and characterisation of mAbs targeting tumour associated glycans expressed on glycoproteins, glycolipids or both.

1.1: B cell biology

Humoral immunity is essential for the host defence against extracellular microbes (including viruses) and microbial exotoxins. The primary cell responsible in generating humoral response are B cells (Twigg, 2005). In addition to making antibodies, B cells perform other critical roles including antigen presentation and regulatory cytokine production. The different B cell subsets have different functions depending upon the inducing stimuli (Naradikian, Scholz, Oropallo, & Cancro, 2014).

1.1.2: B cell lineages

B cells are produced continuously throughout life, initially arising from the fetal liver and then from hematopoietic stem cells in bone marrow (Busslinger, 2004; Dorshkind, 2002; Georgopoulos, 2002). B cells can be separated into two lineages: B-1 and B-2 (Table 1) (Naradikian et al., 2014).

B-1 cells (including B-1a $CD5^+$ and B-1b $CD5^-$ cells) are the earliest B cells (Herzenberg & Tung, 2006) arising from fetal liver and sustained largely as a self-replenishing population in the periphery (Berland & Wortis, 2002; Ghosn, Sadate-Ngatchou, Yang, Herzenberg, & Herzenberg, 2011; Hardy, 2006). B-1 cells participate in innate immunity (Herzenberg & Tung, 2006) and rapidly respond to T-independent antigens and produce low-affinity IgM antibodies. Although marginal zones (MZ) B cells are classified as B-2 cells, they were shown to act like B-1 cells and together with B-1 cells, are known as innate-like B cells (ILBs). In addition to IgM antibody production, B-1 cells and MZ B cells are also actively involved in immune regulation by secreting IL-10 (X. Zhang, 2013). These IL-10 producing ILBs have been classified as innate regulatory B cells (innate Bregs) and they are derived from ILBs that are activated following Toll-like receptor (TLR) or innate microbial stimulation (X. Zhang, 2013). The universal marker for regulatory T cells (Tregs) is the transcription factor, FoxP3. However, there is currently no known master transcription factor that identifies Bregs. IL-10 is an anti-inflammatory cytokine that protects the host from excessive tissue damage during host defence to pathogens and acts as one of the key molecules critically involved in the development and maintenance of immune tolerance and homeostasis (A. C. Anderson et al., 2004; Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). Numerous studies have demonstrated a regulatory role for these innate Bregs in autoimmunity, inflammation and infection (Amu et al., 2010; Fillatreau, Sweenie, McGeachy, Gray, & Anderton, 2002; Gillan, Lawrence, & Devaney, 2005; Mangan et al., 2004; Moore et al., 2001).

B-2 cells arise from the bone marrow and are by far the most predominant B cell population developing in adult bone marrow. Unlike B-1 cells, B-2 cells are relatively short-lived. They do not undergo self-renewal until they are triggered by antigens (Herzenberg & Tung, 2006). In general, B-2 cells recognising T-dependent antigens, and with help from T cells, B-2 cells differentiate into IgG-producing memory B cells (Herzenberg & Tung, 2006) to generate high affinity antibodies with precise antigen specificity (X. Zhang, 2013).

Table 1: Key differences between B-1 and B-2 cells (Herzenberg, 2000; Montecino-Rodriguez & Dorshkind, 2012)

Ontogeny and function	***B-1 cell***	***B-2 cell***
Major roles	Innate immune responses; natural antibodies, T-independent responses	Adaptive immune responses, memory B cells, pathogen specific antibodies, T-dependent and T-independent responses
Atomic locations	Coelomic cavities, mucosal interfaces, spleen	Secondary lymphoid organs, lymphatic blood
Development	Fetal liver, adult bone marrow, self-renewal in periphery	Continuous generation from bone marrow hematopoietic stem cell pool
Subsets	B-1a, B-1b	Transitional (TR), follicular (FO), marginal zone (MZ), germinal centre (GC) and memory B cell
Primary antibody isotypes	IgM, IgA	IgM, IgG

1.1.3: B cell development and the generation of antibody diversity

Humoral immunity is critical in the fight against pathogens. In order to protect an individual against all potential pathogens, the humoral immunity must be capable of producing a large repertoire of B cell receptors (BCRs) (Z. Li, Woo, Iglesias-Ussel, Ronai, & Scharff, 2004). This initial diverse array of BCRs occurs during B cell development (Figure 1.1) and is established via somatic assembly of component variable (V), diversity (D) and joining (J) gene segments at the immunoglobulin heavy

(IgH) and light (IgL) chain loci in a process known as V(D)J recombination (Alt et al., 1984; Dudley, Chaudhuri, Bassing, & Alt, 2005; Tonegawa, 1983). Based upon the status of the immunoglobulin (Ig) chain genes and the expression of different cell surface and intracellular proteins, four major B cell maturation stages have been identified among bone marrow (BM) B cell precursors: pro-B, pre-B, immature and mature naïve B cells (Perez-Andres et al., 2010).

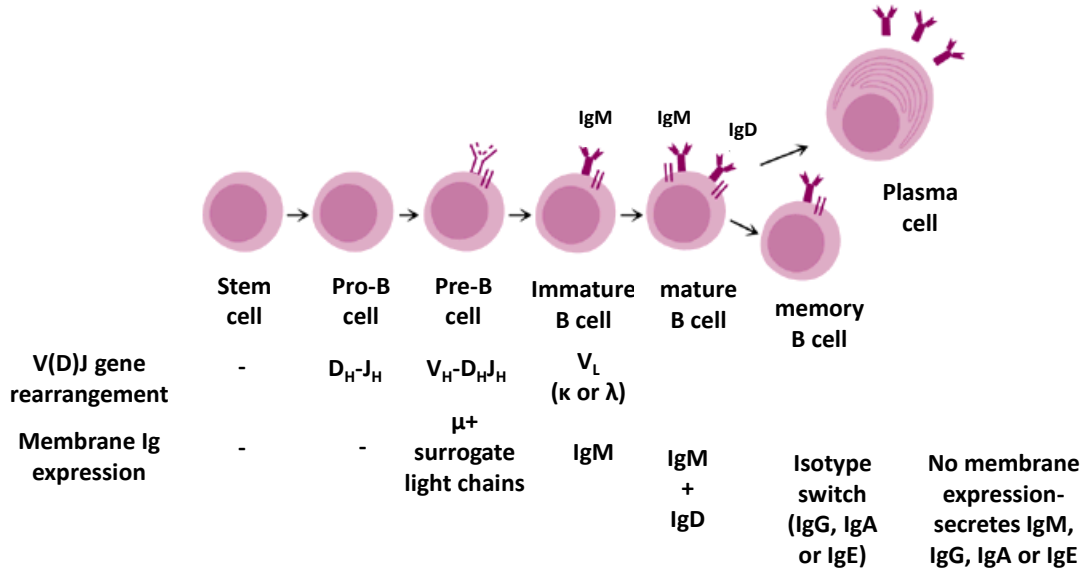


Figure 1.1: B cell development. Dashed lines on the pre-B cell indicate surrogate light chains. The two lines associated with the cell surface heavy chain represent the molecules Igα and Igβ.

During the first step of B cell differentiation, interaction of IL-17 with CD127 expressed on pro-B cell promotes production of several transcriptional factors such as pax-5, PU1, EBF-1 and E2A. These transcriptional factors induce the synthesis of terminal-deoxynucleotidyl-transferase (TdT) and the recombinase activating gene (RAG) products RAG-1 and RAG-2. Both TdT and RAGs are essential to start the V(D)J recombination process which is a well-coordinated set of reactions (Hillion, Rochas, Youinou, & Jamin, 2009; Pelayo, Welner, Nagai, & Kincade, 2006; van Gent et al., 1995). The BCR H chain is consists of V, D and J gene segments whereas the L chain is only comprised of V and J gene segments. The V(D)J recombination process begins by the introducing of double stranded DNA breaks within specific, well conserved recombination signal sequences (RSSs) by RAG-1 and RAG-2 (van Gent et al., 1995). RSSs are located next to every V, D and J gene segment. Each RSS consists of conserved heptamer and nonamer sequences, separated by a spacer sequence that is either 12 or 23 bp in length (Akira, Okazaki, & Sakano, 1987; Tonegawa, 1983) (Figure 1.2). Meanwhile, TdT adds nucleotides to the joining exons randomly to increase the variability of the BCR (Abbas, Lichtman, & Pillai, 2007). Non-homologous end joining (NHEJ) members: Ku70, Ku80, DNA-PKcs, XRCC4, ligase 4 and Artemis then act together to repair the DNA (Bassing, Swat, & Alt, 2002; Taccioli et al., 1993).

Ku70 and Ku80 protect DNA ends generated during V(D)J recombination from unwanted processing and degradation (Boulton & Jackson, 1996), the DNA ends produced by RAG cleavage (Boulton & Jackson, 1996; Cary et al., 1997) and, aid the recruitment of DNA repair and/or DNA damage-sensing proteins (Gottlieb & Jackson, 1993; Lieber, Grawunder, Wu, & Yaneva, 1997; Ramsden & Gellert, 1998). DNA-PKcs have been shown to phosphorylate Artemis, for the formation of coding joints (CJs). The phosphorylated Artemis contained endonuclease activity which is capable of opening DNA hairpins produced by RAGs (Y. Ma, Pannicke, Schwarz, & Lieber, 2002). XRCC4 and DNA ligase 4 work together to ligate the four broken DNA ends generated by RAGs (Figure 1.3a, b and c) (Dudley et al., 2005).

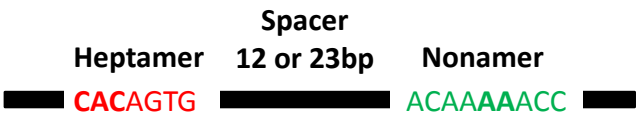


Figure 1.2: Recombination signal sequences (RSSs). RSS consists of a fairly conserved heptamer and nonamer sequence, separated by a spacer element. Heptamer is shown in red and nonamer in green. Conserved nucleotides are shown in bold. The spacer is either 12 or 23bp long.

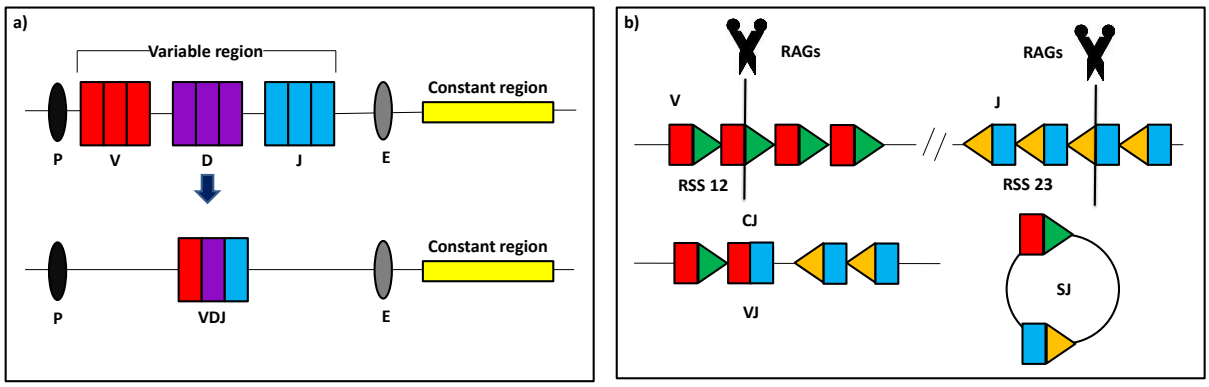


Figure 1.3 a and b: V(D)J recombination takes place within the BCR loci. a) Schematic of a receptor locus. V, D and J segments are found just upstream of the constant region. b) VJ recombination reaction. V segments (red) are flanked by RSSs with 12bp long spacers (green), while the J segments (blue) are flanked by RSS with 23bp long spacers (orange). Breaks are introduced directly between the RSS (heptamer and the nonamer sequences) and coding sequence by RAGs (RAG-1/2), and a CJ is formed between a V and a J segment while the RSS ends are put together to form an SJ within a circular DNA that is later lost. (*p: promoter, E: enhancer; RAG:* recombinase activating gene, *CJ: coding joint, SJ: signal joint*) (Market & Papavasiliou, 2003).

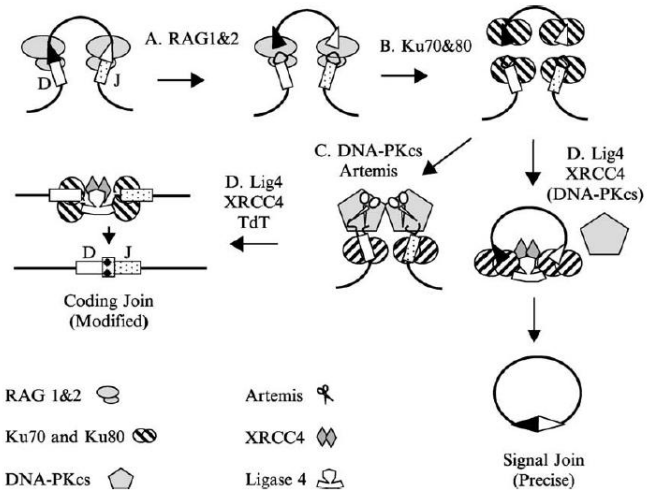


Figure 1.3c: Joining of RAG-mediated DNA double strand breaks. a) RAG-1 and RAG-2 cleavage occurs between RSS and coding segments. b) Ku70 and Ku80 bind to the broken DNA ends. c) DNA-PKcs and Artemis facilitate the opening and processing of covalently sealed hairpin coding ends. d) XRCC4 and DNA ligase 4 (Lig 4) seal the blunt signal ends and processed coding ends to produce precise signal joints and modified coding joints (Dudley et al., 2005).

Upon generation of a functional VDJ exon at IgH, it will be spliced to the constant Igμ exons, generating an Igμ heavy chain. The resulting heavy chain gene product pairs with the invariant surrogate light chain proteins (VpreB and λ5) and the Igα (CD79a) and Igβ (CD79b) signalling proteins to form the pre-BCR complex in a pre-B cell. VpreB and λ5 proteins are substitutes for the Ig light (IgL) chain whose loci have not been rearranged yet at these early stages of B cell development (Espeli et al., 2006; Loffert, Ehlich, Muller, & Rajewsky, 1996). The pre-BCR complex then traffics to the B cell surface (Karasuyama et al., 1994; Pillai & Baltimore, 1987). If the pre-BCR is able to bind in the BM microenvironment (pre-BCR/galectin-1/integrin interactions), the immunoreceptor tyrosine activate motifs (ITAMs) domains of Igα and Igβ are phosphorylated. The downstream signalling of Igα and Igβ rescues these B cells from apoptosis (Espeli, Mancini, Breton, Poirier, & Schiff, 2009; Espeli et al., 2006; Loffert et al., 1996) as well as inducing proliferation of these B cell precursors and rearrangement of the IgL genes (Hess et al., 2001).

The BCR L chain is comprised of V and J gene segments and can be classified into κ and λ L chains. κ L chain locus contains numerous V and J gene segments and a unique C_κ exon. In contrast, λ L chain locus consists of numerous V and J gene segments and 6 to 9 alternative C_λ exons, each with a nearby J_λ gene segment. A DNA rearrangement event join together one V and J segment and its associated C segment to form a single exon. The rearranged L chain sequence is then transcribed by RNA polymerase, generating a L chain primary RNA transcript. The introns in the primary transcript are removed by RNA processing enzymes and the resulting L chain then exits from the nucleus. The L

chain mRNA ultimately being translated into L chain protein, marking the last step of L chain rearrangement, forming the immature B cell with surface expression of IgM and/or IgD (Figure 1.4).

In order to eliminate those cells with auto-reactive BCRs, immature B cells have to pass through a negative selection process. Immature B cells recognising self-antigens in the BM have the opportunity to rearrange their IgL loci to edit their BCR (Abbas et al., 2007; Chen, Prak, & Weigert, 1997; Radic & Zouali, 1996). Immature B cells which unable to generate non-autoreactive BCRs, will be eliminated or become anergic depending on the affinity for self-antigens (Abbas et al., 2007; Espeli et al., 2006). In contrast, immature B cells that pass successfully through this selection will become mature naïve B cells (van Lochem et al., 2004).

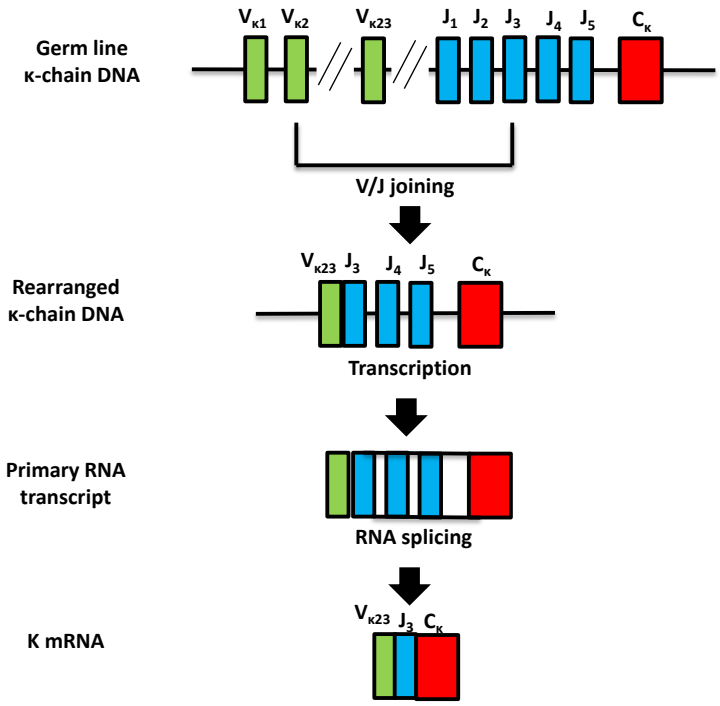


Figure 1.4: The assembly and expression of the κ L chain locus. κ L chain gene rearrangement and RNA processing events required to generate κ light chain protein. In this example, rearrangement joins $V_{κ23}$ and $J_{κ3}$.

The mature naïve B cells then exit the BM via the peripheral blood (PB) and enter the lymph node (LN) at the T cell zone, through high endothelial venules. If the naïve B cells do not encounter antigens, they will exit the LN through lymphatic vessels and die within several days. However, if they encounter their cognate antigens presented by follicular dendritic cells (FDCs) and they are in contact with T cells, they become activated and migrate to the germinal centers (GCs). In the GCs, mature naïve B cells continuously migrate between the dark and the light zones. In the dark zone,

cells undergo rapid proliferation, somatic hypermutation (SHM) of the IgV regions and IgH isotype class switch recombination (CSR) (Allen, Okada, & Cyster, 2007; McHeyzer-Williams & McHeyzer-Williams, 2005). This allows modifications of the genes that encode for low affinity IgMs into high affinity antibodies of the IgG, IgA or IgE isotype (Dudley et al., 2005; MacLennan, 1994). In the light zone, they re-encounter antigen to check that the former events have led to an increased BCR affinity for antigen (Ag) (affinity maturation) (Allen et al., 2007; McHeyzer-Williams & McHeyzer-Williams, 2005).

The constant regions of different immunoglobulin isotypes are encoded by distinct C_H exon clusters, which are organised in the order C_μ, C_δ, C_γ, C_ε and C_α in the IgH locus. In CSR, the C_μ region is replaced by downstream C_γ, C_ε and C_α segments to generate IgG, IgE and IgA antibodies respectively. This confers distinct antibody effector functions without altering the antigen binding variable region (Z. Li et al., 2004). CSR relies on the activity of the *AICDA* gene, which encodes activation-induced deaminase (AID). AID acts by deamination of the cytosine base in the S region of DNA, yielding deoxyuracils (Dudley et al., 2005). AID creates DNA mutations only in single stranded DNA (Dickerson, Market, Besmer, & Papavasiliou, 2003). Subsequently, these single stranded breaks (SSBs) within DNA are converted to double-strand breaks (DSBs), in the upstream (donor) and downstream (acceptor) S regions by unknown mechanisms. The intervening DNA between the S-region forms an R-loop which is subsequently eliminated from the chromosome and the formation of an S-S junction brings the downstream C_H region DNA closer to the $V_HD_HJ_H$ region DNA (Figure 1.5). In the GCs, SHM which inserts mainly point mutations into the antibody variable gene loci leads to the affinity maturation of BCRs (Xu, Zan, Pone, Mai, & Casali, 2012). Like CSR, SHM relies on the activity of AID (Dudley et al., 2005).

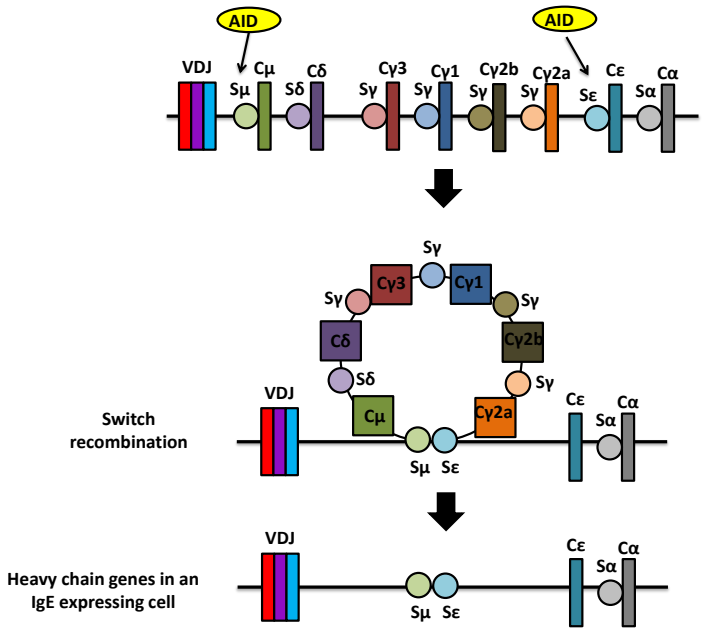


Figure 1.5: Ig class switch recombination (CSR) to IgE. During CSR, activation induced cytosine deaminase (AID) deaminates dC residues in S regions (Sμ and Sε). The intervening DNA between the S-region forms an R-loop which is subsequently eliminated from the chromosome and the formation of an S-S junction brings the downstream C_H region DNA closer to the $V_HD_HJ_H$ region.

1.1.4: B cell activation

When a host is exposed to a microbe or an antigen, either by an infection or by vaccination, naïve B cells will be activated. The process of activation of B cells and the generation of antibody producing cells (plasma cells) consists of distinct sequential phases. Characteristics of the subsequent humoral immune response are fully dependent on the nature of the antigen, the avidity of BCR cross linking and intercellular interactions (Naradikian et al., 2014).

In general, B cell responses follow the two-signal model proposed by Bretscher and Cohn (P. Bretscher & Cohn, 1970) whereby BCR ligation (signal 1) is followed by co-stimulatory activation (signal 2) delivered via other cells or molecules (P. A. Bretscher, 1999).

B cell activation is first triggered by the ligation of BCR to antigen, which initiates a cascade of intracellular signalling followed by the internalisation of antigen for processing and presentation to T cells (Figure 1.6) (Lanzavecchia, 1985). Membrane BCRs are divided into two isotypes, IgM and IgD. The BCR is composed of immunoglobulin domains in the heavy chain linked by a hinge, and a short intracellular domain consisting of just three amino acids: lysine, valine, lysine (KVK). The BCR itself does not contain any signalling motifs but it is linked to the Igα/Igβ heterodimer, which contains ITAMs (Reth, 1989). BCR signalling is initiated by phosphorylation of tyrosines within ITAMs by binding and activating Syk and Lyn (Kurosaki, 1999) and thus resembles signal transduction of many other activating receptors of the immune system (Cambier, 1995).

Humoral responses are classified into T-dependent (T-D) and T-independent (T-I) responses based on the requirement for T cell help (source of signal 2) (Mond, Vos, Lees, & Snapper, 1995). T-D antigens are usually proteins (Parker, Wadsworth, & Schneider, 1980). To stimulate antibody production, T-D antigens must be bound by antigen presenting cells (APCs), endocytosed, processed and presented on major histocompatibility complex (MHC) class II molecules as peptides for helper T cell recognition and activation followed ultimately by T cell-mediated activation of B cells (Mond et al., 1995; Parker et al., 1980). Helper T cell activation leads to the up-regulation of CD40L which is a B cell activating membrane protein. In this manner, helper T cells interact with B cells and activate effectively via CD40-CD40L ligations and secretion of cytokines (Mond et al., 1995). Continuous T-D B cell stimulation within GCs result in B cells undergo somatic hypermutation and affinity maturation, as well as Ig class switching, resulting in the secretion of high affinity IgG antibody for antigen (Mond et al., 1995).

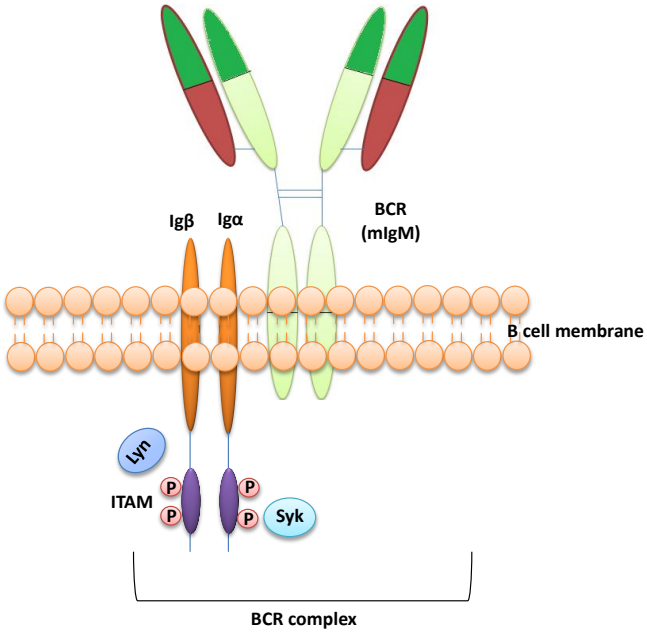


Figure 1.6: B cell receptor (BCR) components and upstream signalling. The BCR is non-covalently coupled to the Igα (CD79a) and Igβ (CD79b) signalling subunits and form BCR complex. Antigen encounter and BCR clustering promotes tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAMs) by Src-family kinases Syk and Lyn. Syk and Lyn activation trigger activation of a downstream signalling cascade leading to cell survival and proliferation.

T-I antigens are usually carbohydrate or lipid antigens. T-I antigens are classified as type I and type II. Type I T-I antigens are intrinsically mitogenic such as lipopolysaccharides (LPS) and CpG which elicit polyclonal B cell activation via TLRs, whereas type II T-I antigens are non-mitogenic repeating unit molecules such as polysaccharides or their haptenated derivatives that extensively crosslink BCRs (Obukhanych & Nussenzweig, 2006; Wortis, Teutsch, Higer, Zheng, & Parker, 1995). Unlike T-D

antigens, T-I antigens do not require T cell help for antibody production. Lipid antigens are internalised in the similar way as T-D antigens, but are presented by the MHC-class-I-like glycoproteins known as CD1d molecules.

The main differences between T-D and T-I responses are: the B cell populations that participate , the antibody isotypes generated, the response duration, the ultimate antibody affinity, and the extent of immunological memory. For T-D responses, B-2 cells, particularly follicular B cells are the major players. In contrast, T-I responses arise primarily from either B-1 cells or B-2 lineage MZ B cells. T-I responses are of short duration and skewed towards IgM production whereas T-D responses produce class switched antibody of IgG isotypes. Moreover, T-D responses display a gradual but profound increase in average antibody affinity, a process known as affinity maturation (Naradikian et al., 2014).

1.1.5: CD1 family

The CD1 family of MHC-class-I-like glycoproteins are non-polymorphic and are encoded by genes residing on chromosome 1 (Martin, Calabi, & Milstein, 1986). Members of the CD1 molecule family can present non-peptide antigens to human T cells (Beckman et al., 1996; Beckman et al., 1994; S. Porcelli, Morita, & Brenner, 1992). The CD1 proteins share sequence homology and overall domain structure with MHC class I molecules, being comprised of a heavy chain with three extracellular domains that are non-covalently associated with Beta-2-microglobulin (β_2m) (D. C. Barral & Brenner, 2007). On the basis of sequence analysis, CD1 molecules fall into three groups: group 1 includes CD1a, CD1b, CD1c; group 2 comprises CD1d and group 3 comprises CD1e (D. Christiansen et al., 2011). Humans have been found to express all CD1 isoforms (D. C. Barral & Brenner, 2007; D. Christiansen et al., 2011). Mouse shares significant sequence homology with human CD1d and it is the only representative of the CD1 family in rodents (Balk, Bleicher, & Terhorst, 1991; Calabi, Jarvis, Martin, & Milstein, 1989). Human CD1 group 1 molecules are widely expressed on APCs such as dendritic cells, Langerhans' cells, activated monocytes, peripheral blood and mantle zone B cells (S. A. Porcelli, 1995). Group 1 CD1 molecules mainly present lipid antigens to a clonally diverse group of T cells (Hughes & Cresswell, 1998; Huttinger, Staffler, Majdic, & Stockinger, 1999; Kang & Cresswell, 2002; Morrice & Powis, 1998) that mediate adaptive immunity to a vast range of microbial lipid antigens (D. C. Barral & Brenner, 2007). Group 2 CD1 molecules (CD1d) present lipid antigens to the invariant natural killer T (iNKT) cells which express an invariant T-cell receptor (TCR) α-chain. This subset of cell responds rapidly following antigen recognition and is a potent effector of innate immunity (D. C. Barral & Brenner, 2007). In contrast to the other CD1 molecules, CD1e is not involved in cell surface presentation of antigen but is involved in intracellular lipid antigen trafficking and processing (de la Salle et al., 2005; De Libero & Mori, 2006).

1.1.6: iNKT cells

NKT cells are a unique subset of T lineage cells, displaying a $CD4^+$ or $CD4^-CD8^-$ double negative phenotype and co-express semi-invariant TCRs and NK-cell receptor (CD161) (Galli et al., 2003; S. Kim, Lalani, Parekh, Wu, & Van Kaer, 2008).

NKT cells can be classified into type I and II based on their TCR repertoire (Godfrey, MacDonald, Kronenberg, Smyth, & Van Kaer, 2004). Type I NKT cells (also known as iNKT cells) are the most prevalent and extensively studied type and they express an invariant TCR, which in the mouse consists of a Vα14Jα18 TCR-α chain, preferentially paired with either Vβ8, Vβ7 or Vβ2 (Godfrey et al., 2004) and in human is Vα24Jα18 TCR-α chain preferentially paired with Vβ11 (Dellabona, Padovan, Casorati, Brockhaus, & Lanzavecchia, 1994; S. Porcelli et al., 1992).

Both human and mouse iNKT cells recognise CD1d (S. A. Porcelli & Modlin, 1999). Natural antigens presented by CD1d to iNKT cells remain unknown. However, α-GalCer, which is a glycolipid composed of α-linked sugar and lipid moieties, originally isolated from marine sponge *Agelas mauritianus* (S. Kim et al., 2008) was found to bind to CD1d and was capable of activating human and mouse iNKT cells (Brossay et al., 1998; Kawano et al., 1997). iNKT cells recognise α-GalCer presented by CD1d molecules on APCs. Upon recognition, α-GalCer activates iNKT cells to rapidly produce large quantities of Th1 (IFN-γ, TNF, IL-4) and Th2 (IL-13) cytokines, as well as other cytokines (IL-2, IL-5, IL-9, IL-10, IL-17, IL-12, TGF-β, GM-CSF) (Kerzerho et al., 2012; Kronenberg, 2005; Matsuda, Mallevaey, Scott-Browne, & Gapin, 2008) and subsequently induces a cascade of activation of various immune competent cells, including dendritic cells (DCs) (Chung, Chang, Kim, & Kang, 2004), natural killer (NK) cells (Metelitsa et al., 2001), B cells (Galli et al., 2003) and $CD4^+$ and $CD8^+$ T cells (Stober, Jomantaite, Schirmbeck, & Reimann, 2003). iNKT cells have been shown to provide help for B cells, increasing B cell proliferation and antibody production via the ligation of CD40 and release of interferon-gamma (IFN-γ) (Galli et al., 2003). Unlike iNKT cells, type II NKT cells express a more diverse TCR repertoire and they recognise CD1d-restricted lipid antigens such as sulfatide but not α-GalCer (Godfrey, Stankovic, & Baxter, 2010). Due to the limited markers available, the type II NKT cells have not been studied as extensively as iNKT cells (Zhao, Weng, Bagchi, & Wang, 2014).

1.1.7: Differentiation of B cells: Memory B cells and plasma cells

Human humoral immunity is capable of conserving specific information regarding the previously encountered pathogens and vaccines, by continuous production of specific antibodies by memory B cells (Yoshida et al., 2010).

Once activated by antigen and receiving a second signal from helper T cells, B cells can develop into two major types of cells, called memory B cells and plasma cells. It is thought that memory B and plasma cells originate from GCs (Thorbecke, Asofsky, Hochwald, & Siskind, 1962). Many reports demonstrated that somatic hypermutation of immunoglobulin genes, affinity maturation, and selection of high avidity antibody variants occurs in the GC (Berek, Berger, & Apel, 1991; Griffiths, Berek, Kaartinen, & Milstein, 1984; Jacob, Kelsoe, Rajewsky, & Weiss, 1991). Memory B cells do not constitutively express any effector function and they require re-stimulation before they can contribute to the effector response. This form of memory is regulated by the immune environment and the quantity of antigen encountered (Yoshida et al., 2010). In contrast, plasma cells persist independently of antigenic stimulation (Manz, Lohning, Cassese, Thiel, & Radbruch, 1998). Plasma cells secrete large amounts of specific antibodies for long periods of time (> 1 year) while remaining as non-dividing B cells in the bone marrow (Manz, Thiel, & Radbruch, 1997; Slifka, Antia, Whitmire, & Ahmed, 1998).

Patients with null mutations in the CD40L gene were found to lack both GC and memory B cells (Agematsu et al., 1998; Facchetti, Appiani, Salvi, Levy, & Notarangelo, 1995; Weller et al., 2001). Similarly, deficiency in the inducible co-stimulator (ICOS) results in impaired GC formation, diminished number of $CD27^{+}$ memory B cells, and lack of durable antibody response against vaccine antigens (Grimbacher et al., 2003; Yong, Salzer, & Grimbacher, 2009). ICOS is a homodimeric protein, which acts by enhancing all T cell responses to antigen, including T cell proliferation, secretion of lymphokines and effective help for antibody secretion by B cells. In addition, ICOS induces the upregulation of IL-10 which is a B cell differentiation factor (Hutloff et al., 1999). Thus, T cell help is essential for formation of long-lived humoral immunity.

Altogether, memory B cells and plasma cells provide a remarkably long-lasting memory. Memory B cells have been detected for more than 50 years after smallpox vaccination (Crotty et al., 2003; Hammarlund et al., 2003) and specific serum antibody secreted by plasma cells have half-lives of more than 50 years (Amanna, Carlson, & Slifka, 2007). Thus, humoral memory provides life-long, specific protection against previously encountered pathogens.

1.2: Antibody

1.2.1: Antibody structure and classes

Antibodies (also known as immunoglobulins) are glycoproteins (~150kDa) secreted by plasma cells and serve as one of the principal effectors of the adaptive immune system (Lipman, Jackson, Trudel, & Weis-Garcia, 2005). Antibodies are composed of four polypeptides, two heavy (H; ~55kDa) and two light (L; ~25kDa) chains (Schroeder & Cavacini, 2010). The antibody L chain can consist of either

a κ or λ chain (W. Wang, Singh, Zeng, King, & Nema, 2007). The H and L chains are held together by disulphide and non-covalent bonds (Figure 1.7) (Lipman et al., 2005).

Antibodies can be classified into five different classes-IgM (μ), IgD (δ), IgG (γ), IgA (α) and IgE (ε), based on their constant Fc regions. There are differences between different classes of antibodies, including size, their ability to fix complement and their Fc receptor (FcR) binding (Schroeder & Cavacini, 2010) (Figure 1.8 and Table 2).

IgM: Naïve B cells express surface membrane monomeric IgM which associates with CD79a and CD79b polypeptide chains that participated in IgM cell signalling. Upon maturation and antigenic stimulation, pentameric IgM is secreted. Generally, each monomeric IgM molecule has low affinity. However, high avidity can be achieved by means of multimeric interactions between the pentameric secreted antibody and the antigen, especially if that antigen contains multiple repeating epitopes. IgM acts by opsonising antigen and activating complement (Schroeder & Cavacini, 2010).

IgD: Similar to IgM, membrane bound IgD is associated with CD79a and CD79b for signalling on naïve B cells. In serum, circulating IgD is found at very low levels with a short serum half-life. To date, the function of circulating IgD remains unclear (Schroeder & Cavacini, 2010).

IgG: IgG is the predominant antibody isotype found in the body with longest serum half-life compared to other antibody isotypes. IgG antibody can be further divided into four subclasses in human (IgG1, IgG2, IgG3 and IgG4) and in mice (IgG1, IgG2a, IgG2b and IgG3). The structural differences between these subtypes are the number and location of interchain disulphide bonds as well as the length of the hinge region (S. J. Wang & Bourguignon, 2006). The IgG subclasses exhibit different functional activities including complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC) as well as apoptosis. In ADCC, an IgG antibody binds to its target on tumour cells via its antigen binding site followed by interacting with specific Fc-gamma receptors (FcγRs) expressed on immune effector cells via Fc region (Strome, Sausville, & Mann, 2007). In human, IgG1 and IgG3 bind to all three FcγR classes whereas IgG4 binds only to FcγRII and III, with significantly weaker binding compared to IgG1 and IgG3. IgG2 binds only FcγRII (Schroeder & Cavacini, 2010). In mice, IgG2a and IgG2b bind to FcγRI, FcγRIII and FcγRIV whereas IgG3 to FcγRI and IgG1 to FcγRIII (Bruhns, 2012). In CDC, activation of the complement cascade is an important mechanism for the clearance of opsonised pathogens. CDC involves the interaction of at least 2 IgG to a C1q molecule leading to the formation of C3b molecule which in turn induced the formation of the C5-C9 membrane attack complex (MAC) that can insert into tumour cell membrane and cause cell lysis. The most effective human antibody isotype for stimulating the classic complement cascade

were identified to be IgG3 followed by IgG1. IgG2 is not very effective in stimulating CDC whereas IgG4 is unable to induce CDC (Strome et al., 2007). In mice, the most potent IgG isotypes able to activate the complement system are IgG2a, IgG2b and IgG3 (Nimmerjahn, 2014).

IgA: IgA can be found at high levels at mucosal surfaces and in secretions, including saliva and breast milk. At the mucosa, IgA is a dimer. IgA is critical at protecting mucosal surfaces from toxins, virus and bacteria by neutralisation or by prevention of binding to the mucosal surface (Schroeder & Cavacini, 2010).

IgE: IgE is a very potent immunoglobulin. It is associated with hypersensitivity, allergic reactions as well as parasitic worm infections. IgE binds to the FcεRI which can be found on mast cells, basophils, Langerhans cells and eosinophils with high affinity. IgE has a much lower affinity for FcεRII (CD23) which is expressed on mast cells, basophils, Langerhans cells, eosinophils as well as B cells, NK cells and platelets (Schroeder & Cavacini, 2010).

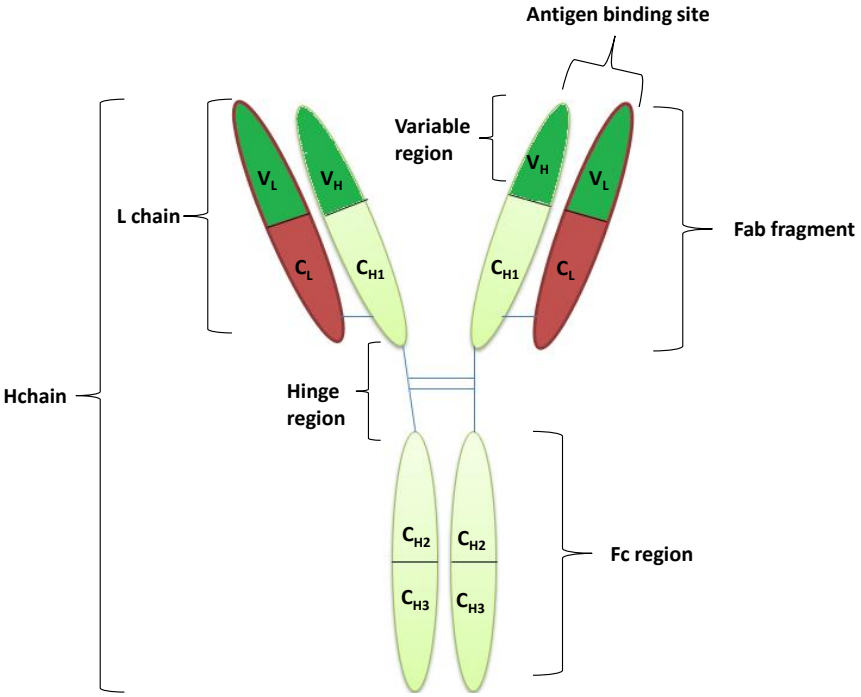


Figure 1.7: Structure of an antibody molecule. (*L chain: light chain; H chain: heavy chain; V_L: light chain variable region, V_H: heavy chain variable region, C_L: light chain constant region; C_H: heavy chain constant region*)

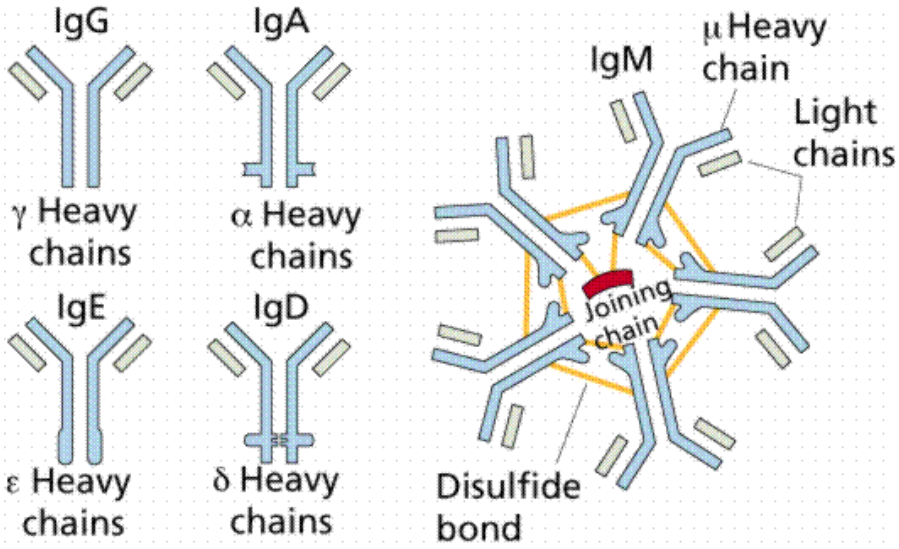


Figure 1.8: The five classes of human antibodies (Purves, Orians, & Heller, 1995).

Table 2: Properties of human IgG subclasses (Vidarsson, Dekkers, & Rispens, 2014).				
	IgG1	IgG2	IgG3	IgG4
Molecular mass (kDa)	146	146	170	146
Mean adult serum level (g/l)	6.98	3.8	0.51	0.56
Relative abundance (%)	60	32	4	4
Half-life (days)	21	21	7-21	21
Antibody response to:				
Proteins	++	+/-	++	++
polysaccharides	+	+++	+/-	+/-
Allergens	+	-	-	++
Complement activation				
C1q binding	++	+	+++	-
Fc receptors (FcRs)				
FcγRI	+++	-	++++	++
$FcγRIIa_{H131}$	+++	++	++++	++
$FcγRIIa_{R131}$	+++	+	++++	++
FcγRIIb/c	+	-	++	+
$FcγRIIIa_{F158}$	++	-	++++	-
$FcγRIIIa_{V158}$	+++	+	++++	++
FcγRIIIb	+++	-	++++	-
FcγRn (at pH < 6.5)	+++	+++	++/+++	+++

1.2.2: Antigen binding site

The function of antibodies involves specific binding to antigens and activation of other components of the immune system to fight pathogens (Sela-Culang, Kunik, & Ofran, 2013). The N-terminus of the antibody light and heavy chains associate to form antibody variable domains (antigen-binding domains) (Schroeder & Cavacini, 2010). The six hypervariable loops within the variable domains of antibodies, commonly termed complementarity determining regions (CDRs), are the epitope binding sites (Sela-Culang et al., 2013). The C-terminus of the two heavy chains fold together to form the Fc constant domains (Schroeder & Cavacini, 2010), which are responsible in mediating effector activation (Figure 1.9a) (Sela-Culang et al., 2013). Somatic hypermutation in the antibody variable regions allows antibodies to recognise a huge repertoire (~10^9) of antigens and the constant Fc domains allow the antibodies to interact with FcRs on the surface of innate immune cells (Hayes et al., 2014).

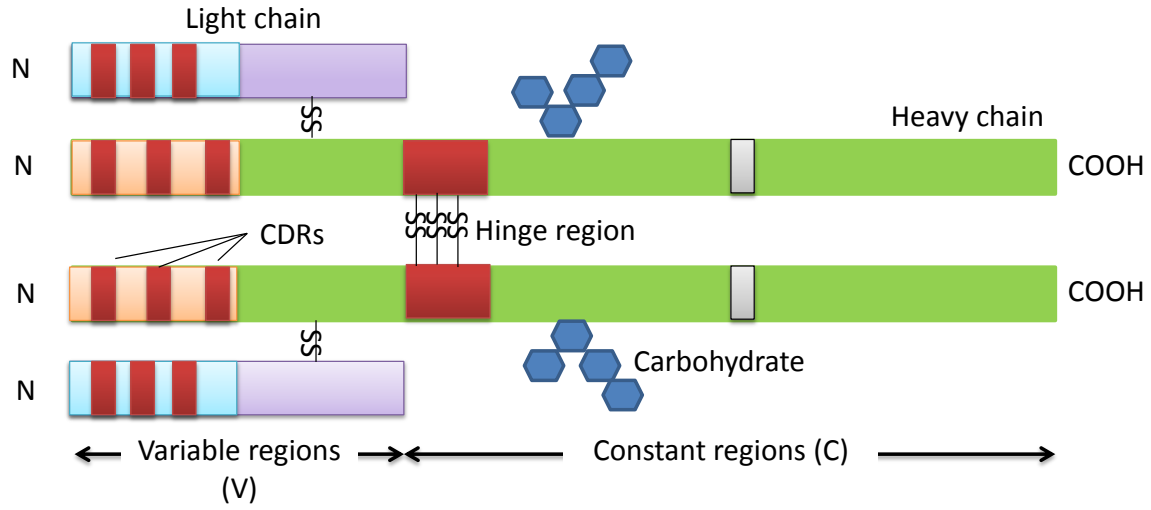


Figure 1.9a: Schematic polypeptide chain structure of an IgG molecule.

1.2.3: The role of N-glycans in IgG biology

The N-linked glycans present on the conserved Asn-297 residues of IgG molecules are essential for antibody structure and function (Hayes et al., 2014). The conserved Asn-297 is buried between the CH2 domains of the Fc region (Harris, Shire, & Winter, 2004; Kroon, Baldwin-Ferro, & Lalan, 1992). In addition to glycosylation of the two canonical Asn-297 residues in the Fc regions, N-linked glycans are also found in 20% of the Fab regions in some human IgG. These glycans include bi-antennary structures that are hyper-galactosylated, fucosylated, and extensively sialylated (Mimura, Ashton, Takahashi, Harvey, & Jefferis, 2007). Up to 36 different IgG N-glycan structures have been reported (Wormald et al., 1997) (Figure 1.9b). Various studies have clearly shown that proper glycosylation is critical for correct functioning of antibodies (A. Wright & Morrison, 1997). For example, changes of

Fc glycosylation of IgG1 and IgG3 result in the ineffective binding of the antibodies to C1q molecule, the first component of the complement cascade (A. Wright & Morrison, 1997); ineffective binding to human FcγRI; and sensitivity to more proteases than their corresponding wild type IgGs (Tao & Morrison, 1989). Furthermore, loss of Fc glycosylation affects the antibody conformation (Tao & Morrison, 1989) as well as disrupting the structural integrity of the Fc region, which is required for optimal binding to the Fc receptor (Hayes et al., 2014).

The sugar composition of the oligosaccharide is also crucial in antibody effector functions, including ADCC and CDC (Jefferis, 2009a, 2009b, 2009c; Raju, 2008; Reichert, 2009, 2010, 2011; Strohl & Knight, 2009). It has been shown that removal of core fucose at Asn-297 of the Fc region exhibit enhanced binding affinity to activating FcγR and more potent ADCC (Kanda et al., 2007; Okazaki et al., 2004; Shields et al., 2002). Due to the significant impact on immune effector function, various studies have been carried out to generate afucosylated mAb therapeutics. Genetic elimination of fucosyltransferase 8 (FUT8) in Chinese hamster ovary (CHO) cells has been shown as a successful approach for the prevention of core fucosylated IgG (Yamane-Ohnuki et al., 2004). In addition, overexpression of β(1,4)-N-acetylglucosaminyltransferase III (GnTIII) which eliminates the substrate for fucosyltransferase within CHO cells has also been developed (Umana, Jean-Mairet, Moudry, Amstutz, & Bailey, 1999). Several studies have demonstrated that changes in the terminal galactosylation of IgGs may affect the CDC activity of the antibodies (Hodoniczky, Zheng, & James, 2005; Raju, 2008). Hodoniczky and Zheng et al. showed that degalactosylation of Rituximab reduced the CDC activity of the mAb by 50%, when compared to the native mAb (Hodoniczky et al., 2005).

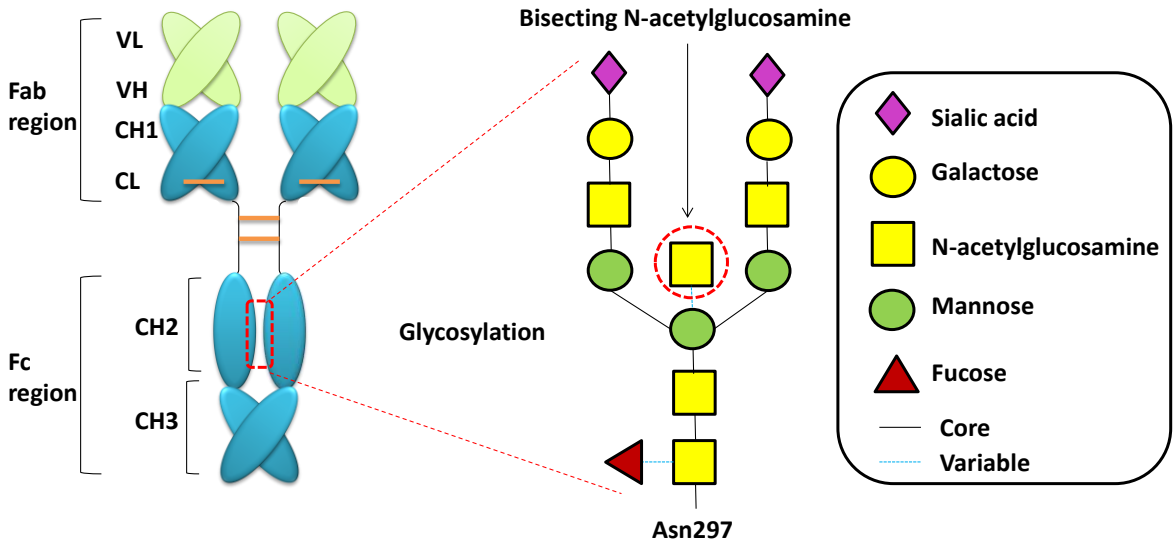


Figure 1.9b: IgG structure and the N-linked glycans. IgGs are ~150kDa tetramers comprising pairs of identical heavy and light chains linked by disulphide bonds (orange bars). The heavy chains contain a variable domain (VH) and three constant domains (CH1, CH2 and CH3), whereas the light chains contain a variable domain (VL) linked to a single constant domain (CL). Human IgG molecules, particularly IgG1 and IgG3 can bind to antigen on the target cell surface and interact with Fc receptors on effector cells via the Fc domain of antibodies to cause target cell destruction through ADCC and ADCP. Interaction of antigen bound antibodies with complement component, C1q may support killing via CDC. Engineering of Fc domain glycosylation allows the improvement of the effector functions of the antibodies. For example, mutating conserved asparagine 297 (Asn297) prevents glycosylation to generate aglycosylated antibodies lacking effector functions. ADCC can be enhanced by increasing the bisecting N-acetylglucosamine in the Fc carbohydrate or by eliminating fucose (Kubota et al., 2009) [adapted from (Chames, Van Regenmortel, Weiss, & Baty, 2009)].

1.3: Generation of monoclonal antibodies

1.3.1: Hybridoma technology

MAbs are identical antibodies that bind to the same epitope of an antigen and are derived from a single B cell clone (Little, Kipriyanov, Le Gall, & Moldenhauer, 2000). In 1975, mAbs were first introduced and generated in mice using hybridoma technology by Kohler and Milstein (Figure 1.10) (Kohler & Milstein, 1975).

The generation of hybridomas begins with immunisation of animals (usually mice) with the antigen of interest. Throughout the immunisation process, the anti-sera from the immunised animals are used to develop and validate the screening procedure. Once a good humoral response (titre) has been achieved in the immunised animals, splenocytes of the immunised animals are fused with an immortal myeloma cell line lacking the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene. The hybridomas are grown *in vitro* in hypoxanthine-aminopterin-thymidine (HAT) medium, which can only sustain HGPRT positive cells. Aminopterin blocks the pathway that allows for

nucleotide synthesis. Therefore, only hybridomas survive in HAT medium as they inherited immortality from the myeloma cells and HGPRT from the primary B cell clones. The hybridomas secrete their own individual antibody into the culture medium. After fusion, each individual clone can be separated by serial dilution into multi-well tissue culture dishes so that each well contains only a single-cell-hybridoma. The cell culture medium from different wells are screened for specific antibody activity and the desired wells are expanded and recloned until a mAb has been generated (F. Li, Vijayasankaran, Shen, Kiss, & Amanullah, 2010; Little et al., 2000; Liu, 2014).

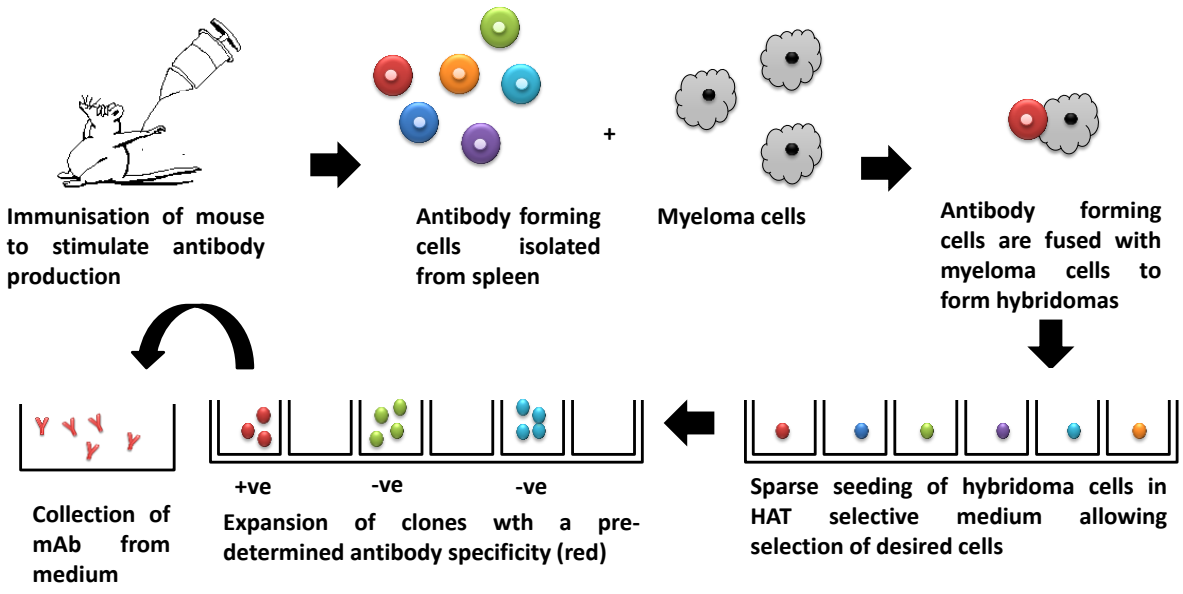


Figure 1.10: Principle steps in the production of a hybridoma. Splenocytes (spleen cells) are harvested from immunised animals (usually mice) and then fused with myeloma cells to generate hybridoma cells. Hybridomas are grown in HAT selective medium. The hybridomas are propagated in a highly diluted state so that colonies deriving from single hybridoma can be isolated. MAbs secreted into medium are screened and the clones which produce desired mAbs are identified and expanded.

1.3.2: Phage display technology

In 1985, George Smith introduced the alternative method of generating mAbs by using the phage display technology (Figure 1.11) (G. P. Smith, 1985). Phage display technology allows the presentation of large peptide and protein libraries including antibodies on the surface of filamentous phage, which leads to the selection of most antibodies recognising targets with high affinity and specificity.

The technology involves the introduction of DNA encoding millions of different antibody fragments into a location in the genome of the phage capsid proteins in order to generate a phage library containing approximately 10^{10} variants (Schofield et al., 2007). Due to limitations of the *E.coli* folding

machinery, only antibody fragments like single chain fragment variable (scFv), fragment antigen binding (Fabs), the variable heavy domain of humans (dAbs), which bind specifically without a corresponding light chain variable domain are used in phage display technology (Figure 1.11a) (Hoet et al., 2005; Holt, Herring, Jespers, Woolven, & Tomlinson, 2003; Hust & Dubel, 2005). The encoded antibody fragments are displayed on the phage surface as a fusion product with one of the phage coat proteins. The antigen is immobilised on a solid surface such as nitrocellulose, magnetic beads, column matrices, plastic surfaces like polystyrene or 96 well microtitre plates and incubated with antibody phage of the antibody gene libraries. Antibody phage particles that do not or only bind weakly to the antigens are removed by stringent washing, a process known as 'biopanning'. Antibody phage particles that bind to antigens specifically are eluted, amplified and reselected 6-10 times to ensure specificity. (Schirrmann, Meyer, Schutte, Frenzel, & Hust, 2011).

a)

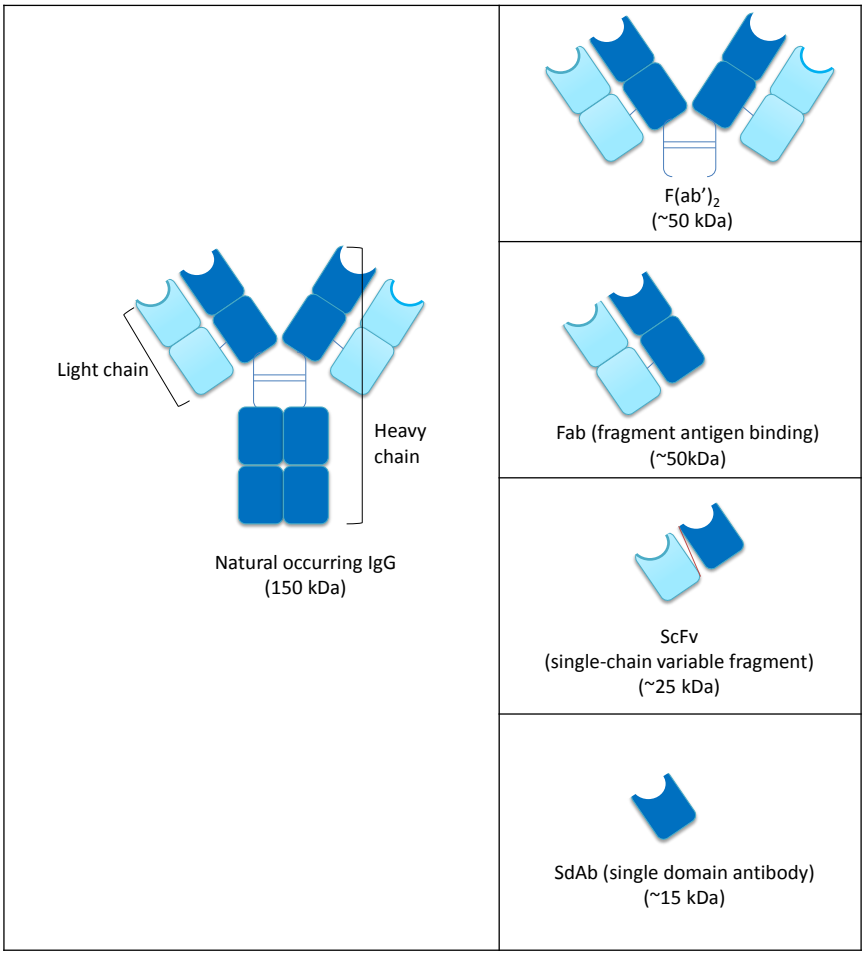


Figure 1.11a: A selection of engineered antibody fragments. A schematic representation of different antibody formats showing the classical IgG molecule alongside antibody fragments that can be generated: $(F(ab')_2$, Fab, ScFv and SdAb).

b)

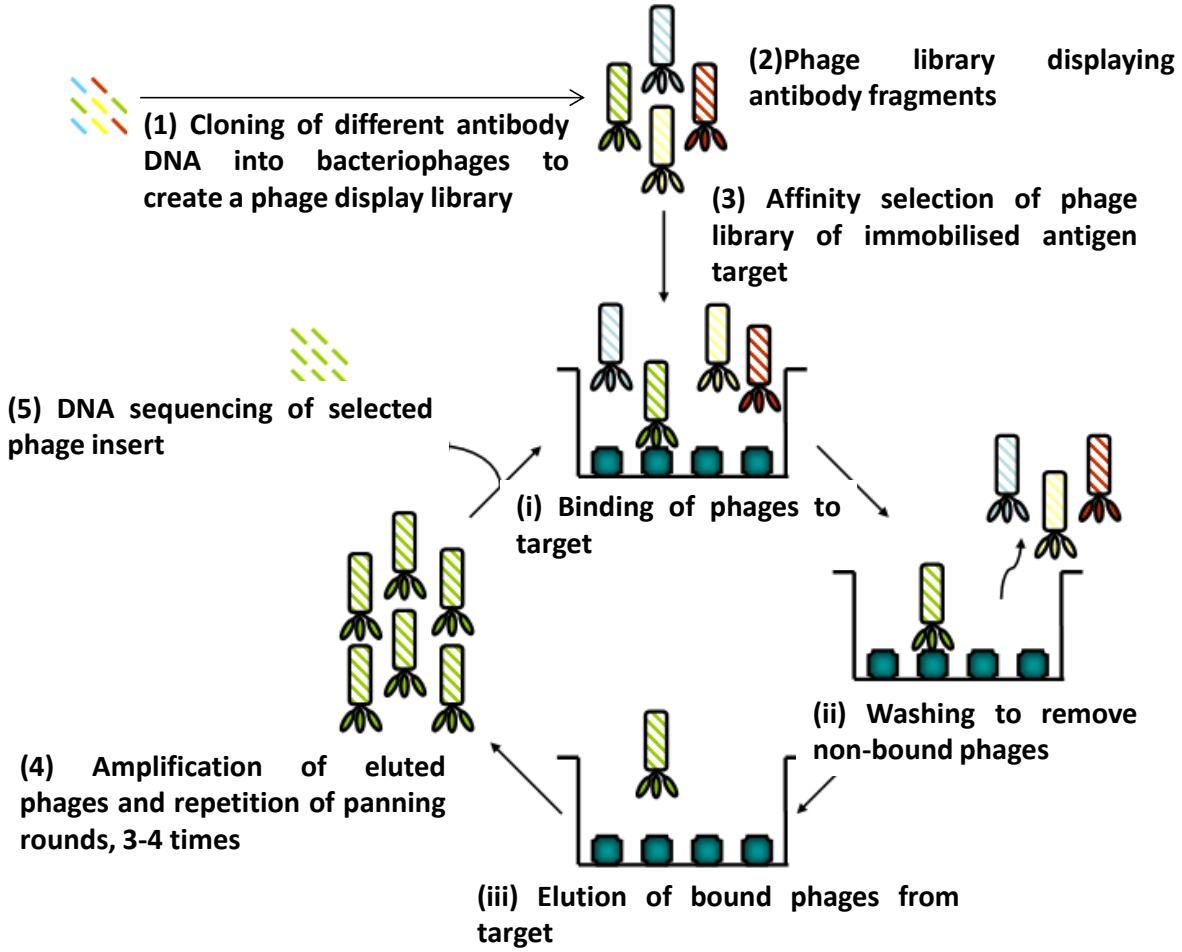


Figure 1.11b: Principle steps in phage display technology. (1) DNA encoding millions of different Ab fragments (Fab, scFv or sdAb) is cloned into the genome of filamentous bacteriophage linked to one of the phage coat protein genes; (2) Each DNA variant is packed into separate phage particle, and the Ab fragment displayed on the phage protein coat; (3) Phage displaying Fab, scFv, sdAb that bound to the target analyte are selected using biopanning cycles of (i) binding, (ii) washing, and (iii) elution; (4) Eluted phages are reinfected into *E.coli* cells and amplified for further rounds of affinity selection; (5) Clones from the enrinched library are characterised for binding properties using appropriate techniques (Yun et al., 2009).

Traditionally, phage display technology had been used to generate antibody fragments targeting protein antigens. However, with the realisation that tumour associated carbohydrate antigens (TACAs) are important targets for active specific and passive immunotherapy, many attempts of generating anti-carbohydrate-specific antibody fragments using phage display technology were attempted (Deng et al., 1994; Fukuda, 2012; Kieber-Emmons et al., 1999; van Kuppevelt, Dennissen, van Venrooij, Hoet, & Veerkamp, 1998; Yuasa et al., 2010) but they were difficult to isolate due to their low affinity (dissociation constant of 10^{-6} to 10^{-7} M) (Mao et al., 1999; Schoonbroodt et al., 2008). Generation of anti-carbohydrate mAbs by utilising mimotope (molecular mimicry of

carbohydrate antigens) as a means to convert the T-I responses to T-D responses appeared to be an attractive alternative. Epitope mapping techniques have demonstrated that peptides with YPY motif mimics mannose (Oldenburg, Loganathan, Goldstein, Schultz, & Gallop, 1992; J. K. Scott, Loganathan, Easley, Gong, & Goldstein, 1992), WRY mimics α-1,4-glucose (Murai et al., 1985), WLY mimics Le^y (Hoess, Brinkmann, Handel, & Pastan, 1993), YRY mimics the major C polysaccharide α-2,9-sialic acid (Westerink, Giardina, Apicella, & Kieber-Emmons, 1995) and W/YXY motif mimics neolactoseries structures Le^x, SLe^x, Le^a, SLe^a and Le^b (Agadjanyan et al., 1997; Imberty et al., 1995). However, most studies have shown that mimotopes are effective in eliciting high level of IgM responses, but not IgG responses (Kieber-Emmons et al., 1999) unless they were coupled to other protein carriers such as tetanus toxoid (TT) (Hafner et al., 2005) and keyhole limpet hemocyanin (KLH) (W. Li et al., 2013).

1.3.3: Enhancement of antigen immunogenicity

To generate mAb with high antigen specificity, affinity and of the IgG isotype, immunisation schemes are crucial (immunogens/antigens and adjuvants) (Grimaldi & French, 1995).

Antigens are defined as molecules that can elicit an immune response. In general, protein antigens are more immunogenic compared to carbohydrate, lipid or nucleic acid antigens. The immunogenicity of antigen molecules can be enhanced by conjugation to immunogenic carrier molecules, emulsification in adjuvants, or both (Grimaldi & French, 1995).

a) Immunogenic carrier

In general, glycolipid antigens are poorly immunogenic. However, it is possible to improve antigen immunogenicity by conjugation to highly immunogenic protein carriers such as KLH (Kantele et al., 2011), TT and bovine serum albumin (BSA) (Huang et al., 2013).

Ragupathi *et al.* demonstrated the immunisation of groups of mice with Globo-H conjugated to the KLH protein carrier in the presence of a potent immunological adjuvant (QS21), resulted in the induction of anti-sera against the Globo-H positive tumour cell line, MCF-7. IgM antibody titres were generally higher than IgG titres throughout the immunisation regime. These anti-sera were highly effective in inducing CDC (Ragupathi *et al.* 1997). Subsequently, Huang et al. compared the immune responses generated in mice immunised with Globo-H conjugated to different carrier proteins such as KLH, diphtheria toxoid, cross reactive material (CRM) 197 (DT), TT and BSA in the presence or absence of α-GalCer C34 adjuvant. Globo-H conjugated to DT in the presence of α-GalCer C34 (Globo-H-DT/C34) appeared to be the best combination. In addition, comparison between Globo-H-DT/C34 with the phase III trial vaccine, Globo-H-KLH/QS21 showed that Globo-H-DT/C34 induced higher titre IgG serum antibodies (Huang et al., 2013).

Although protein carriers have been shown to aid in generating higher immune response against low immunogenicity antigens, there is also evidence showing that due to their own high immunogenicity, protein carriers may interfere with immune responses to polysaccharide antigens (Herzenberg, Tokuhisa, & Herzenberg, 1980).

b) Adjuvant

Adjuvants are agents that have been used for more than 70 years to enhance immune responses to specific antigens that are weakly immunogenic (Jennings, 1995; Stills, 2005). Adjuvants were initially introduced as agents to sustain, promote and augment cell mediated antibody immune responses. However, evidence has shown that adjuvants can also affect titre, duration, isotype, and avidity of antibody (Jennings, 1995). To date, diverse classes of compounds have been assessed as adjuvants including mineral salts, microparticles, liposomes, microbial products, saponins, emulsions, cytokines and polymers (Guy, 2007).

Adjuvants act by a combination of multiple mechanisms including the formation of depot, activation of APCs, induction of cytokines and chemokines leading to recruitment of immune cells, enhancement of antigen uptake and presentation, and promoting antigen transport to draining lymph nodes (Awate, Babiuk, & Mutwiri, 2013; Cox & Coulter, 1997).

'Depot effect': The 'depot' effect has been recognised as the classical mechanism of action of adjuvants. The 'depot' effect of adjuvants prevents the antigen from dilution and rapid degradation as well as sustains release of small quantities of antigen over a long period of time (Stills, 2005). In 1969, Siskind and Benacerraf demonstrated by localising and releasing low-dose antigen over long period of time, the adjuvant aids in the stimulation of B cells with high affinity receptors. In contrast, when B cells are exposed to high-dose of antigens, B cells with medium- and low-affinity receptors would be stimulated (Siskind & Benacerraf, 1969). Emulsions (Freund, Casals, & Hosmer, 1937), aluminium compounds (Gupta, 1998), microparticles (Eldridge et al., 1991), liposomes and immune stimulating complexes (ISCOMs) (Gomez-Vargas, Rosenthal, McDermott, & Hortelano, 2004) are the most commonly used depot adjuvants in the induction of antibodies.

'Activation of APCs': Activation of APCs is essential for the induction of adaptive immune responses. Activation of APCs results in the increased expression of MHC II and co-stimulatory molecules (CD83 and CD86) which ultimately lead to the enhanced ability of APCs in inducing T lymphocyte activation and differentiation (Coyle & Gutierrez-Ramos, 2001). Examples of adjuvants which induce APCs maturation to enhance adaptive immunity are: LPS, liposomes, Freund's complete adjuvant, CpG-ODN, alum, MF59, AS04, TLRs and α-GalCer (Copland et al., 2003; De Becker et al., 2000; De Smedt

et al., 1996; Fujii, Shimizu, Smith, Bonifaz, & Steinman, 2003; Kaisho & Akira, 2002; Shah et al., 2003).

CD40-CD40L: CD40 costimulatory molecule, which is a member of the tumour necrosis factor (TNF) receptor superfamily (Hassan, Sorensen, Olsen, & Pedersen, 2014), plays a central role in humoral immunity (Zarnegar et al., 2004). The expression of CD40 molecule can be found on various APCs, including DCs, B cells, monocytes and macrophages as well as epithelial cells, endothelial cells and platelets (Bourgeois, Rocha, & Tanchot, 2002; Khalil & Vonderheide, 2007; Lee et al., 2005; Luqman et al., 2008; J. Ma et al., 2012). The engagement of CD40 ligand (CD40L/CD154) expressed on T helper cells to CD40 molecule on a B cell results in the enhancement of B cell survival, proliferation, GC formation, memory B cell development as well as antibody isotype class switching and affinity maturation (Foy et al., 1994). Anti-CD40 mAb has been reported to mimic T cell help (CD40L engagement) in mice and human and showed powerful effects on T helper and cytotoxic cell responses (Diehl et al., 1999; Dullforce, Sutton, & Heath, 1998; French, Chan, Tutt, & Glennie, 1999; Ninomiya, Ogasawara, Kajino, Takada, & Kida, 2002; Sotomayor et al., 1999).

1.3.4: Recombinant technology

The development of therapeutic mAbs has resulted in substantial benefits to public health. To date, more than 20 unconjugated therapeutic antibodies have received regulatory approval for the treatment of cancers and autoimmune diseases (Jiang et al., 2011).

In the 1980s, the success of hybridoma technology enabled the generation of rodent mAbs against human pathogens and cells. However, evidence showed that they had limited clinical utility due to their short half-life in serum (Faller et al., 1990). IgG affinity for FcRn is species specific, which explains the low affinity of human FcRn to mouse IgG results in the rapid clearance of murine IgG in humans (W. Wang, Wang, & Balthasar, 2008). In addition, they were suboptimal in eliciting Fc effector functions (Reichert, 2010) and were highly immunogenic in humans (Breedveld, 2000).

These issues led to the advent of recombinant engineering techniques which permitted the construction of mAbs customised for the binding site but with modification of the antibody constant region, resulting in the development of chimeric, humanised and human antibodies that are less immunogenic, more efficient at promoting desired effector functions and have longer half-lives (Figure 1.13) (Breedveld, 2000).

Chimeric mAbs are less immunogenic in humans as they are 70% human and possess a fully human Fc portion, which allows them to interact with human effector cells and the complement cascade (Chames et al., 2009). Later, with the rapid advancement of antibody engineering techniques, it

became possible to generate 'humanised' mAbs which are 85-90% human, by replacing the hypervariable loops of a fully human antibody with the hypervariable loops of the murine antibody of interest, via a method known as CDR grafting (Jones, Dear, Foote, Neuberger, & Winter, 1986) (Figure 2). Another major improvement came with the development of transgenic 'humanised' mice which were created by replacing the entire mouse IgG repertoire with a human repertoire (Lonberg, 2008). Upon immunisation, these humanised mice produce *in vitro* matured human IgGs, circumventing the need for additional affinity maturation. Moreover, they directly lead to full length IgG, which is often the preferred format for therapy (Chames et al., 2009).

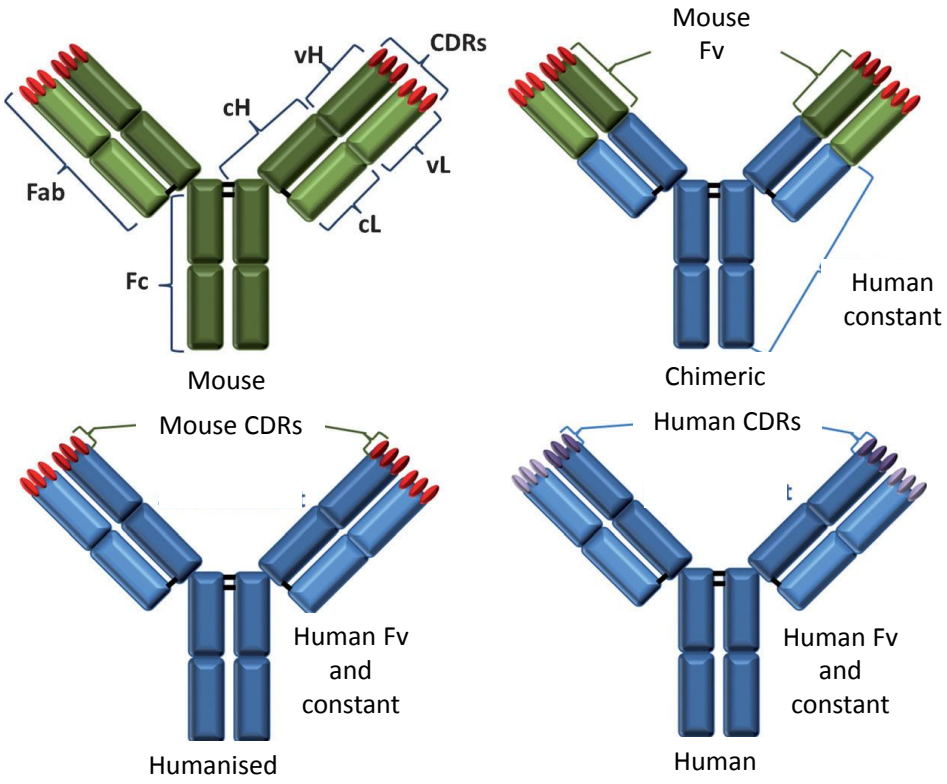


Figure 1.13: Murine (green), chimeric, humanised and human (blue) antibodies. The antibody subdomains are marked, including Fab, Fc, heavy-chain variable (vH), light-chain variable (vL), heavy chain constant (cH), light chain constant (cL) and the complementary determining regions (CDRs) (Chari, Miller, & Widdison, 2014).

1.4: Antibody-based immunotherapy of cancer

Specific recognition and elimination of malignant cells by antibodies were proposed over a century ago (Weiner, Murray, & Shuptrine, 2012). The development of antibody-based therapies for cancer has been the focus of considerable interest for decades. To date, many therapeutic mAbs have been developed and have proven useful in cancer therapy (Table 3).

Therapeutic mAbs mediate tumour cell killing via several mechanisms: direct action of the mAb (through receptor blockade and induction of apoptosis), immune-mediated cell killing (ADCC, ADCP and CDC), delivery of drugs or cytotoxic agents and as immune checkpoint inhibitors (A. M. Scott, Wolchok, & Old, 2012).

Table 3: FDA approved therapeutic monoclonal antibodies for use in cancer therapy

MAb	***Target***	***Isotype***	***Cancer Indication***
Gemtuzumab	CD33	Humanised IgG4-toxin	Acute myeloid leukemia
^{90}Y-ibritumomab	CD20	Murine IgG1-radionuclide	B cell lymphoma
Rituximab	CD20	Chimeric IgG1	Non-Hodgkin lymphoma
Trastuzumab	HER2	Humanised IgG1	Breast cancer
Alemtuzumab	CD52	Humanised IgG1	Chronic lymphocytic leukemia
Cetuximab	EGFR	Chimeric IgG1	Colorectal cancer
Bevacizumab	VEGFA	Humanised IgG1	Colorectal, breast and lung cancers
Panitumumab	EGFR	Human IgG2	Colorectal cancer
Ofatumumab	CD20	Human IgG1	Chronic lymphocytic leukemia
^{131}I-tositumomab	CD20	Murine IgG1-radionuclide	B cell lymphoma
Ramucirumab	VEGFR2	Human IgG1	Colorectal cancer, NSCLC
Pertuzumab	HER-2	Humanised IgG1	Breast cancer
Denosumab	RANKL	Human IgG2	Giant cell tumour of the bone, prostate cancer, breast cancer
Obinutuzumab	CD20	Humanised IgG1	Chronic lymphocytic leukemia
Blinatumomab	CD19, CD3	Bispecific	B cell precursor acute lymphoblastic leukemia
Nivolumab	PD-1	Human IgG4	Melanoma, Squamous NSCLC

Siltuximab	IL-6	Chimeric IgG1	Renal cancer, Prostate cancer
Ipilimumab	CTLA-4	Human IgG1	Melanoma
Pembrolizumab	PD-1	Humanised IgG4	Metastatic melanoma
Dinutuximab	GD2	Chimeric IgG1	Neuroblastoma

a) Neutralising membrane proteins

Antibodies may target tumour cells by engaging to the surface antigens overexpressed in cancers (Weiner et al., 2012). It was recognised that mAbs with high specificity can evoke tumour cell death by blocking receptor signalling, regulating tumour cell growth and promote tumour cell apoptosis (Strome et al., 2007). Bevacizumab is a humanised IgG1 mAb directed against vascular endothelial growth factor (VEGF). Binding of VEGF to VEGF-1 and VEGF-2 receptors on vascular endothelial cells stimulates angiogenesis. Binding of Bevacizumab to VEGF blocks the interactions between VEGF and VEGF-1/2 receptors and therefore inhibits new vessel growth. Panitumumab is a human IgG2 mAb. It binds to the extracellular domain of epidermal growth factor receptor (EGFR). Upon ligand binding, EGFR dimerises and activates downstream signalling pathways that ultimately lead to cell proliferation and tumour growth. Panitumumab acts as competitive antagonist of EGF and transforming growth factor-α (TGF-α) (Strome et al., 2007). Trastuzumab and pertuzumab are both humanised IgG1 anti-HER-2 mAbs that act by inhibiting the dimerization of HER-2/neu and HER-2/HER-3 receptors respectively, thus blocking the intracellular signalling pathways of HER-receptor positive tumours (Capelan et al., 2013).

b) Antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP)

ADCC is the killing of an antibody-coated target cell by a cytotoxic effector cell, characterised by the release of the cytotoxic granules and cell death-inducing molecules such as perforin, granzymes, tumour necrosis factor (TNFα) and reactive oxygen intermediates (Figure 1.14a). ADCP involves the phagocytosis of an antibody-coated target cell by a macrophage (Figure 1.14b). ADCC and ADCP are induced via the interaction of target-bound antibodies with FcRs, glycoproteins present on the effector cell surface that bind to the Fc regions of immunoglobulins. Effector cells that are capable of inducing ADCC and ADCP include natural killer (NK) cells, macrophages, neutrophils and eosinophils (Herter et al., 2014; Teillaud, 2012).

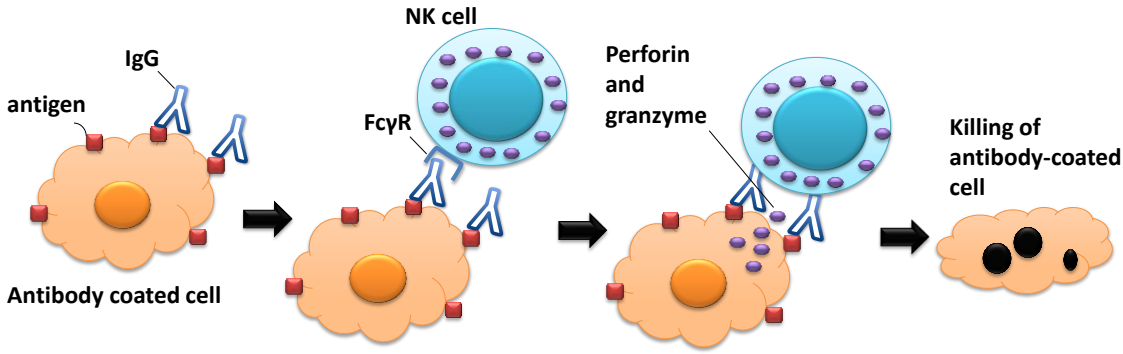


Figure 1.14a: Antibody dependent cellular cytotoxicity (ADCC). IgG molecules bind to antigen on a target cell. A NK cell recognises the Fc region of the IgG molecule via FcγR. Once the NK cell engages with the IgG molecule, the NK cell is activated and releases cytokines (IFN-γ) and cytotoxic granules (perforin and granzymes) that enter the target cell and promote cell death via apoptosis.

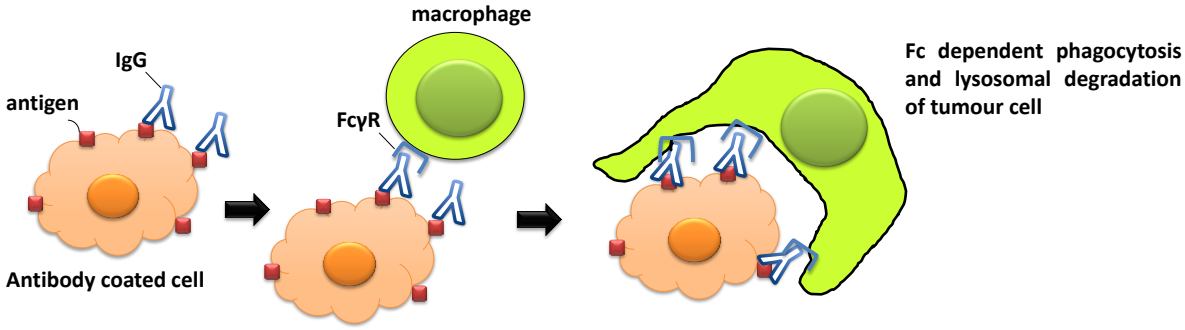


Figure 1.14b: Antibody dependent cellular phagocytosis (ADCP). IgG molecules bind to antigen on a target cell. A macrophage recognises the Fc region of the IgG molecule via FcγR. Once the macrophage engages with the IgG molecules, the macrophage gets activated and phagocytosed the antibody-coated tumour cell.

c) Fcgamma receptors (FcγRs)

(i) Human FcγRs

Most hematopoietic cells, except most T cell subsets, express FcγRs (Nimmerjahn & Ravetch, 2008). Human FcγRs can be classified into three types, FcγRI, FcγRII (a, b and c) and FcγRIII (a and b). FcγRI (CD64), FcγRIIa (CD32a), FcγRIIc (CD32c) and FcγRIIIa (CD16a) are activation FcγRs whereas FcγRIIb (CD32b) is an inhibitory receptor. The function of FcγRIIIb (CD16b) remains unknown. Activation FcγRs possess ITAMs (Ra, Jouvin, Blank, & Kinet, 1989) which are essential for cell activation whereas inhibitory FcγRs contain immunoreceptor tyrosine-based inhibition motifs (ITIMs) which are required for negative regulation of cell activation, degranulation, proliferation, endocytosis and phagocytosis (Daeron, Jaeger, Du Pasquier, & Vivier, 2008; K. G. Smith & Clatworthy, 2010) (Figure 1.15).

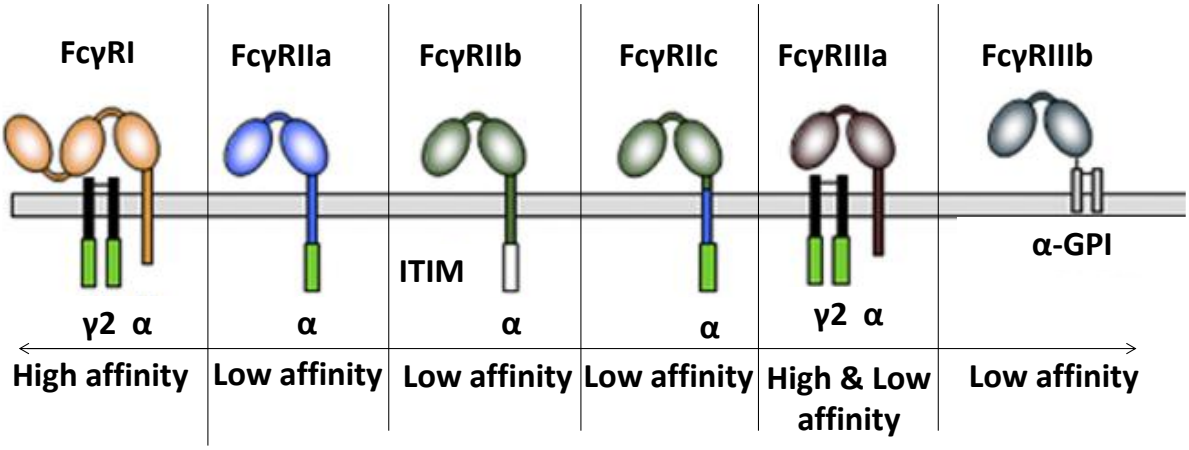


Figure 1.15: Human FcγRs. Schematic representation of human FcγRs at the cell membrane (gray bar) and their association with FcγR-chain dimer (black). Green boxes represent ITAMs and white boxes ITIMs (Bruhns, 2012).

FcγRI can be found on monocytes/macrophages, DCs, and are inducibly expressed on neutrophils (Ravetch & Kinet, 1991) and mast cells (Okayama, Kirshenbaum, & Metcalfe, 2000). FcγRIIa is expressed on all myeloid cells but not on lymphocytes; FcγRIIb can be found on circulating B cells, basophils, macrophages, DCs (Veri et al., 2007), and weakly on monocytes and neutrophils (Magnusson, Engstrom, Jacob, Ulfgren, & Kleinau, 2007; Veri et al., 2007). FcγRIIc is expressed on NK cells (Bruhns et al., 2009), monocytes and neutrophils (van der Heijden et al., 2012). FcγRIIIa on is highly expressed on NK cells, monocytes and macrophages; FcγRIIIb on neutrophils (Meknache, Jonsson, Laurent, Guinnepain, & Daeron, 2009).

FcγRs can be classified into low or high affinity receptors. In general, both low and high affinity FcγRs are able to bind antigen-antibody immune complexes or IgG aggregates with high avidity, but only high affinity FcγRs can bind IgG as monomers. Stimulation of immune effector cells via FcγRs may result in the activation of a variety of effector functions, including ADCC, ADCP and CDC (Bruhns, 2012; Bruhns et al., 2009; Jefferis & Lund, 2002). Each of these human FcγRs recognises the Fc domain of human IgG antibody subclasses with different affinities, which exhibits unique activation profile for the different human IgGs (Table 4).

Table 4: Human IgG- Fc recognition specificity for effector ligands (Jefferis & Lund, 2002).					
IgG isotype	FcγR expression	IgG1	IgG2	IgG3	IgG4
FcγRI	Monocytes, macrophages	3+	-	4+	2+
FcγRIIa	Monocytes, macrophages, neutrophils, eosinophils, platelets	+	-	2+	-
FcγRIIb	B cells, monocytes, macrophages	2+	ND	2+	+
FcγRIIIa	Macrophages, large granular lymphocytes, natural killer cells, γδ T cells	+	-	+	-
FcγRIIb	Neutophils, eosinophils	+	-	+	-
Complement activation	C1q	3+	+	3+	-

(ii) Mouse FcγRs

Mouse FcγRs can be classified into four types, FcγRI, FcγRIIB, FcγRIII and FcγRIV. FcγRI, FcγRIII and FcγRIV are activation FcγRs whereas FcγRIIB is an inhibitory receptor. All mouse activation FcγRs bind to IgG2a and IgG2b but FcγRI is the only activation FcγR, which binds to IgG3 and FcγRIII to IgG1 (Figure 1.16) (Bruhns, 2012).

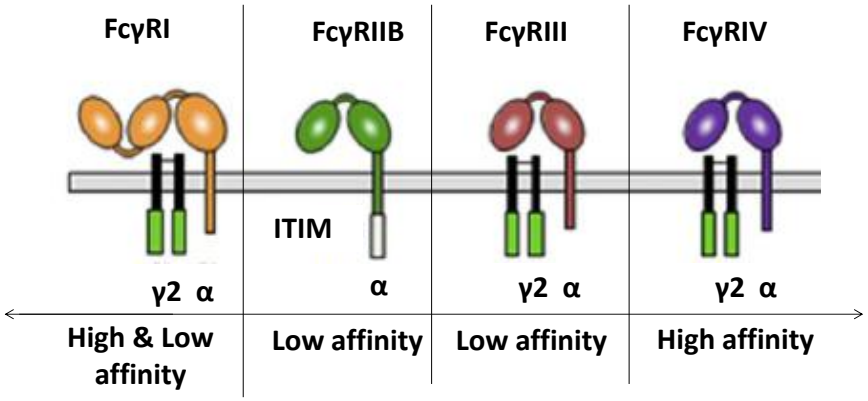


Figure 1.16: Mouse FcγRs. Schematic representation of mouse FcγRs at the cell membrane (gray bar) and their association with FcγR-chain dimer (black). Green boxes represent ITAMs and white boxes ITIMs (Bruhns, 2012).

1.4.1: Effector cells of ADCC

Human NK cells ($CD56^{dim}$ $CD16^{+}$) are thought to be the key player in inducing ADCC (Banks, Kinsey, Clements, & Hildreth, 2002). Human NK cells appear to express relatively high levels of FcγRIIIa but do not co-express other classes of FcRs. FcγRIIIa is a transmembrane receptor (can be found on NK cells and macrophages) that is associated with an activating molecule involved in signal transduction known as the FcR γ chain. In contrast, FcγRIIIb that can be found on neutrophils is expressed as a phosphatidyl inositol glycan-linked receptor that does not associate with the FcR γ chain and is not involved in tumour cell killing (Teillaud, 2012).

Monocytes and macrophages express all three classes of FcγRs. However, only low levels of FcγRIIIa are expressed on monocytes. The FcγRIIIa level increases when monocytes mature into macrophages. It has been noted that activated macrophages are $FcγRIIIa^{high}FcγRIIb^{low}$ thus favouring cell activation whereas quiescent macrophages are $FcγRIIIa^{low}FcγRIIb^{high}$, providing high threshold for cell activation (Ravetch & Lanier, 2000; Teillaud, 2012).

1.4.2: Complement dependent cytotoxicity (CDC)

The complement system, a central innate system, efficiently protects the host from pathogen attack through a wide range of cellular responses (Morgan, 1999; Walport, 2001). The complement system is composed of three distinct protease cascades known as classical, mannose-binding lectin (MBL) and alternative pathways. More than 30 soluble plasma proteins and membrane proteins are involved in the complement system (Figure 1.17) (Zhou, Hu, & Qin, 2008).

The classical and MBL pathways are initiated by the binding of recognition proteins to specific targets. The classical pathway is initiated by the binding of one IgM or two IgGs as well as C-reactive protein and serum amyloid P protein to target antigens. The binding of these antibodies to antigens

then allows the binding of C1q molecule to the Fc regions of antibodies; initiating the classical activation cascade. The MBL pathway is triggered by the binding of MBL to an array of carbohydrates (specifically mannose and fucose residues) on the surface of microbial pathogens (Walport, 2001). In contrast to classical and lectin pathways, the alternative pathway is capable of autoactivation via a process termed 'tickover' of C3 molecule. Tickover occurs spontaneously at a rate of ~1% of total C3 per hour (Muller-Eberhard, 1988; Thurman & Holers, 2006), thereby identifying any potential pathogens.

All three pathways converge at the C3 level, which allows C3b and C5 proteins to target the pathogens and ultimately leads to the polymerisation of C9 by C5b-8 binding to form MAC with a diameter of 5-10nm (Morgan, 1999). MAC inserted into the pathogen cell membrane directly induces targeted cell lysis through subsequent influx of ions and water that leads to lethal colloid-osmotic swelling (Zhou et al., 2008). In addition to the formation of MAC for target cell lysis, opsonisation of target cells with opsonins (C1q, C3b and C4b) for phagocytosis by myeloid cells and generation of anaphylatoxins C3a and C5a to trigger chemotaxis of effector cells are part of complement activation (Derer, Beurskens, Rosner, Peipp, & Valerius, 2014).

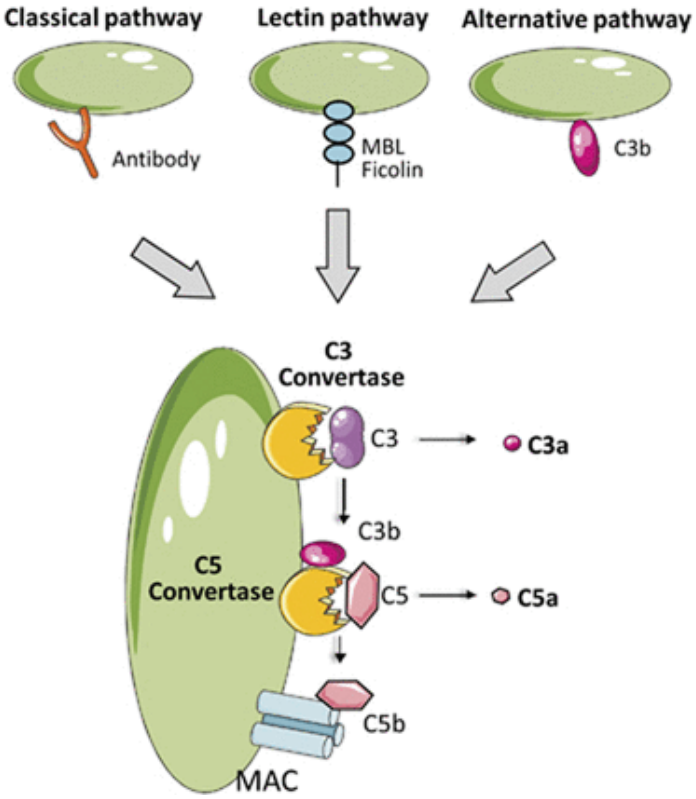


Figure 1.17: Complement activation pathways. Complement activation can be initiated via 3 pathways (classical, lectin and alternative). The classical pathway is triggered by antibody-bound bacteria; the lectin pathway specifically recognises a microbial sugar moiety with mannose-binding lectin (MBL) or ficolins and the alternative pathway is activated spontaneously. Upon initiation,

signalling pathways are activated and all pathways converge at the C3 level by the formation of C3 convertases. Incorporation of another C3b molecule into existing C3 convertases generates C5 convertases. C5 convertases cleaves C5, resulting in the release of C5a and the sequential deposition of C5b and C9, which leads to the formation of membrane attack complex (MAC).

1.4.3: Membrane complement regulatory proteins (mCRPs)

Normal tissues express mCRPs to protect themselves from being destroyed by locally activated complement. Tumours over-express mCRPs (Campbell & Morgan, 1985; Morgan, 1989; Muller-Eberhard, 1988). MCRPs on solid tumours can be divided into three types, CD46 (membrane cofactor protein; MCP), CD55 (decay-accelerating factor; DAF) and CD59 (Fishelson, Donin, Zell, Schultz, & Kirschfink, 2003). CD46 is a transmembrane protein with a cytoplasmic domain (Fishelson et al., 2003). CD46 acts as cofactor for factor-I (fI) but does not contain decay accelerating activity (Kojima et al., 1993). CD46 can be generated by alternative splicing and then expressed in different isoforms on the same cell (Post et al., 1990). CD55 is a glycosyl phosphatidylinositol (GPI)-anchored integral membrane protein (Davitz, Low, & Nussenzweig, 1986; McHeyzer-Williams & McHeyzer-Williams, 2005) involved in inducing the decay of C3/C5 convertases with preferential inactivate the classical pathway C4b/C2a convertase (Nicholson-Weller, March, Rosen, Spicer, & Austen, 1985). CD59 plays a role as a last line of defence upon initiation of MAC assembly (Meri et al., 1990). It acts by inhibiting C8 and C9 polymerisation (Nose, Katoh, Okada, Kyogoku, & Okada, 1990). CD59 is GPI anchored to the cell surface like CD55 and it is detected on all circulating cells and most tissues. Many groups observed that the more mCRPs that were expressed on tumour cells, the more they were resistant to killing by complement (Weichenthal, Siemann, Neuber, & Breitbart, 1999). It is believed that the level of expression of each mCRPs may reflect a stage in differentiation of specific tumour cells. Thus, poorly differentiated neoplastic cells expressed low or no mCRPs, whereas differentiated neoplastic cells expressed higher mCRPs levels. For example, Koretz et al have reported that the expression of CD59 increased from poorly differentiated to highly differentiated colorectal carcinoma cells (Koretz, Bruderlein, Henne, & Moller, 1993).

1.4.4: Apoptosis/ 'Programmed cell death'

Antibody-mediated tumour cell killing is frequently associated with ADCC and CDC. In addition to ADCC and CDC, antibodies are capable of eliciting apoptosis either directly or indirectly within tumour cells via activation of death receptors and blockade of ligand-receptor growth or survival pathways (Ludwig, Pereira, Zhu, Hicklin, & Bohlen, 2003).

Apoptosis or programmed cell death is a form of controlled cell death, in response to a variety of stimuli. Apoptosis is a normal component of the development and health of multicellular organisms. Apoptotic cells display distinctive morphology during the apoptotic process. Typically, the cell begins

to shrink following the cleavage of lamins and actin filaments in the cytoskeleton. The breakdown of chromatin in the nucleus often leads to nuclear condensation. In addition, the translocation of phosphatidylserine from the inside of the cell to the outer surface promotes phagocytosis of apoptotic cells by macrophages. The end stages of apoptosis are often characterised by the appearance of membrane blebs and small vesicles known as apoptotic bodies (Majno & Joris, 1995).

Death receptors are cell surface receptors that when they engage to ligands such as Fas ligand, TNF-α and TRAIL, they transmit pro-apoptotic signal in cells. Example of death receptors are: the tumour necrosis factor receptor-1 (TNFR1), TNF-related apoptosis-inducing ligand receptor (TRAIL)-R1 and TRAIL-R2 as well as Fas (Ashkenazi & Dixit, 1998; Nagata & Golstein, 1995). These receptors each carry a death domain which allows the recruitment of various apoptotic proteins to the receptor, necessary for transmission of the apoptotic signal following receptor activation (Ashkenazi & Dixit, 1998). The notion of targeting these receptors on tumours with mAbs which act by inducing apoptosis is attractive since they are frequently expressed on tumour cells and can act directly to trigger apoptosis (Ludwig et al., 2003).

1.4.5: ADC

Traditional cancer chemotherapy is often accompanied with severe side effects to cancer patients. MAbs recognising cancer cells offer an alternative tumour-selective treatment approach. Antibody-drug conjugates (ADCs) are composed of a therapeutic mAb chemically linked to a potent cytotoxic drug. By combining the specific antigen targeting capability of a mAb with the cancer-killing ability of a cytotoxic drug, ADCs selectively targeting tumour cells thus improving the therapeutic index of chemotherapeutic agents by minimising the peripheral damage to neighbouring healthy tissues (Chari et al., 2014).

The development of ADCs involves a critical understanding of target antigen, internalisation efficacy of the antibody, drug potency as well as the stability of the linker connecting the drug and antibody. Currently, there are two ADCs that are approved and many being tested in clinical trials (Table 5, Figure 1.17).

Table 5: Antibody drug conjugates (ADCs) currently approved/ in clinical trials (Chari et al., 2014)			
FDA-approved	**Cytotoxic drug**	**Antigen**	**Cancer**
Ado-Trastuzumab emtansine (Kadcyla)	DM1	HER2	Breast cancer
Brentuximab vedotin (Adcetris)	MMAE	CD30	HL/ALCL
Phase III			
Inotuzumab ozogamicin (CMC-544)	Calicheamicin	CD22	ALL
Gemtuzumab ozogamicin (CMA-676)	Calicheamicin	CD33	AML
Phase II			
SAR3419	DM4	CD19	B cell malignancies
RG7593	MMAE	CD22	B cell malignancies
RG7596	MMAE	CD79b	B cell malignancies
Glembatumumab vedotin (CDX-011)	MMAE	GPN MB	Breast cancer, melanoma
PSMA-ADC	MMAE	PSMA	Prostate cancer
Phase I			
Lorvotuzumab mertanisine	DM1	CD56	SCLC
IMGN529	DM1	CD37	B cell malignancies
IMGN853	DM4	FRα	Solid tumours
IMGN289	DM1	EGFR	Solid tumours
SAR566658	DM4	CA6	Solid tumours
BT-062	DM4	CD138	Multiple myeloma
BAY 94-9343	DM4	Mesothelin	Solid tumours
AMG 595	DM1	EGFRvIII	Gliomas

AMG 172	DM1	CD27L	ccRCC
SGN-CD19A	MMAF	CD19	NHL/ALL
AGS-22ME	MMAE	Nectin 4	Solid tumours
RG 7450	MMAE	STEAP1	Prostate cancer
RG 7458	MMAE	MUC16	Ovarian cancer
RG 7599	MMAE	Napi2b	NSCLC, ovarian cancer
MLN 0264	MMAE	GCC	GI malignancies
SGN-CD33A	PBD	CD33	AML
MDX-1203	Duocarmycin	CD70	NHL, RCC
Labetuzumab-SN-38	SN-38	CD66e	CRC
IMMU-132	SN-38	Trop-2	Epithelial cancers
Milatuzumab Doxorubicin	Doxorubicin	CD74	Multiple myeloma

HL, Hodgkin's lymphoma; ALCL, anaplastic large cell lymphoma; NHL, Non-Hodgkin's lymphoma; AML, Acute myelogenous leukemia; SCLC, Small cell lung cancer; ccRCC, Clear cell renal cell carcinoma; RCC, Renal cell carcinoma; ALL, Acute lymphoblastic leukemia; NSCLC, Non-small cell lung cancer; GI, Gastrointestinal; CRC, colorectal carcinoma

a) ADC-The antibody

The key function of the antibody of an ADC is to bind to antigens on the target cell and thus concentrate the linked cytotoxic drug at the tumour site. Ideally, the antigen is expressed in high copy numbers (>10^5/cell) and homogeneously on the tumours. In addition, the desired antibody affinity to the antigen is crucial. High antibody affinity (K_d< 1nM) has been shown to be ideal for good tumour localisation. However, some *in vivo* studies suggested that antibodies with lower antibody affinity are able to penetrate solid tumours to a greater extent (Rudnick et al., 2011). Another important factor of an ADC is its efficiency to internalise into a target cell. The internalisation process, also known as receptor-mediated endocytosis, is dependent on the nature of the antigen, with some growth factor receptors such as the EGFR known to rapidly internalised (Orth, Krueger, Weller, & McNiven, 2006).

b) ADC-The linker

One of the major challenges in the development of effective but safe ADCs has been the generation of suitable chemical linkers. Due to the complexity of linker chemistry, a number of ADCs that initially showed promising preclinical data have failed in the clinic due to the released of the cytotoxic anticancer agent linked to the antibody prematurely before arriving at the target cell. Gemtuzumab ozogamicin, which is an anti-CD33 mAb conjugated to calicheamicin was withdrawn from the market due to the unstable linker, which released 50% of bound drug before reaching the tumour, thus increasing toxicity (Petersdorf et al., 2009; Ravandi, 2011).

There are two types of chemical linkers being used to generate ADCs, including cleavable linkers (acid-labile linkers, protease cleavable linkers and disulfide linkers) and non-cleavable thioether linkers (Panowksi, Bhakta, Raab, Polakis, & Junutula, 2014). The nature of the chemical linkers is crucial. In particular, once ADCs engage their antigens, they internalise into target cells and some intracellular release mechanism cleaves the chemical linkers to release the active cytotoxic drugs. Acid-labile linkers (e.g., gemtuzumab ozogamicin) relied on the acidic pH value (~pH5) of the lysosomes, whereas the protease cleavable linkers (e.g., brentuximab vedotin) relied on lysosomal enzymes, such as peptidases and esterases, for cleavage (Chari et al., 2014). Disulfide linkers (e.g., IMGN-901) are selectively cleaved in the cytosol in the reductive intracellular environment (Erickson et al., 2010; Panowksi et al., 2014). Non-cleavable linkers release cytotoxic drugs after antibody degradation in the lysosome (Teicher & Chari, 2011).

ADCs are designed to bind to antigen positive tumour cells. Therefore, the cytotoxic effect of ADCs might be impaired if the target antigens are expressed on tumours in a heterogeneous fashion. To eradicate tumours with homogeneous and heterogeneous expression of the tumour antigens, ADCs with 'bystander' effect have been studied extensively. The 'bystander effect' is a process by which neighbouring cancer cells are killed when free drug is released into the tumour environment by dying cells (Kovtun et al., 2006). Disulfide linkers are used to allow ADCs a 'bystander effect' (Kovtun et al., 2006). Anti-CanAg mAb conjugated to maytansinoid DM1 (Kovtun et al., 2006) and anti-mesothelin mAb conjugated to maytansinoid tubulin inhibitor DM4 (Golfier et al., 2014) via disulphide linkers are examples of ADCs with bystander effect.

c) ADC-The cytotoxic drug

Doxorubicin is a cytotoxic drug belonging to the anthracycline family, synthesised from a natural source produced by *Streptomyces actinobacteris*. Doxorubicin acts by inhibiting DNA religation, leading to DNA double-strand breaks (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). As a chemotherapeutic agent, it has been used in the treatment of several cancers such as

haematological malignancies, carcinomas and soft tissue sarcomas. Doxorubicin has been conjugated to chimeric BR96 mAb (cBR96; anti-Lewis y mAb) via acid-labile linker (Bouchard, Viskov, & Garcia-Echeverria, 2014). Although Doxorubicin-cBR96 had showed compelling and promising preclinical data compared to free Doxorubicin, it demonstrated limited clinical therapeutic activity as well as a narrow safety profile in a randomised phase II breast cancer study. It was suggested that cBR96 mAb itself also contributed to the toxicity (Tolcher et al., 1999).

Calicheamicin is a DNA-alkylating agent produced by *Micromonospora echinospora ssp. Calichesis* (Carter & Senter, 2008). Calicheamicin acts by causing DNA double-strand breaks (Ricart & Tolcher, 2007). Gentuzumab ozogamicin is an ADC formed by conjugating Calicheamicin to humanised anti-CD33 IgG4 antibody; hP67.6 via acid-labile linker. It was the first ADC approved by FDA in 2000 for the use as second line therapy for the treatment of relapsed acute myelocytic leukemia (Bouchard et al., 2014). However, in 2010, Gemtuzumab ozogamicin was voluntary withdrawn from the market due to safety concerns coupled with limited clinical benefit in a confirmatory post-approval phase III clinical trial (Bouchard et al., 2014).

Maytansine is a benzoansamacrolide that was first isolated from the bark of the Ethipan Shrub Maytenus ovatus (Morris Kupchan et al., 1972). Maytansine acts by binding to tubulin, resulting in the suppression of microtubule dynamics, causing cells to arrest in the G2/M phase of the cell cycle, and ultimately cell death by apoptosis (Lopus et al., 2010). Two maytansine derivatives, emtansine (DM1) and ravtansine (DM4) have been widely used in combination with cleavable and non-cleavable linkers (Bouchard et al., 2014). Trastuzumab emtansine is a (HER2)-targeted antibody conjugated to DM1 via a stable thioether linker (Lewis Phillips et al., 2008). It has been approved as a second line treatment for breast cancer patients that have previously failed to respond to trastuzumab and taxane-based chemotherapy (Bouchard et al., 2014). In a phase III clinical trial of patients with HER2 positive metastatic breast cancer and central nervous system metastases, Trastuzumab emtansine was shown to improve median overall survival by six months and progression-free survival by 50% compared to lapatinib/capecitabine with limited systemic toxicity (Krop et al., 2015).

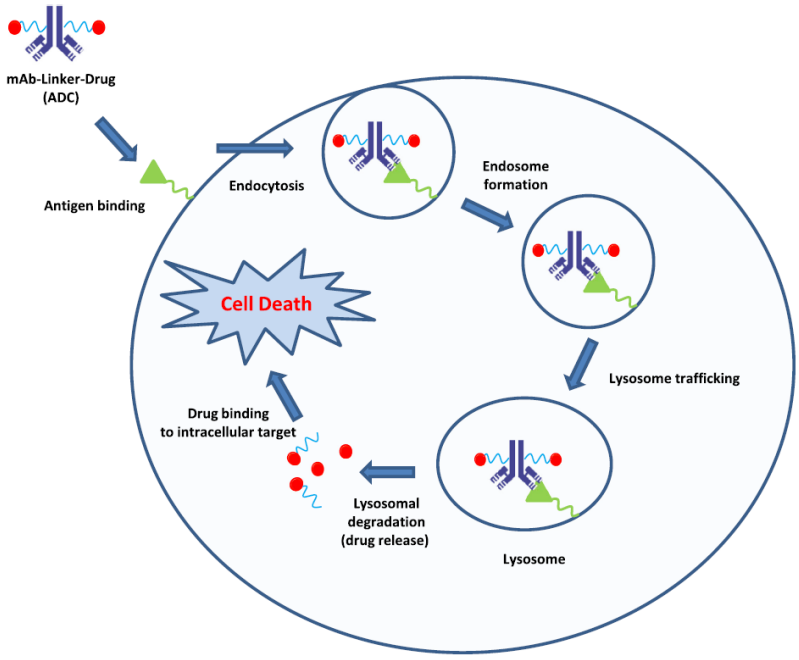


Figure 1.17: Schematic representation of the process associated to the mechanism of action and biological activity of antibody drug conjugates (ADCs) (Bouchard et al., 2014).

d) Immune checkpoint inhibitors

The clinical development of checkpoint blocking mAbs is based on the understanding that in addition to co-stimulatory signal, co-inhibitory signals regulate antigen-specific immune responses. Blockade of co-inhibitory molecules such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein-1 (PD-1) have been showed to enhance T cell responses against tumours (Callahan & Wolchok, 2013; Intlekofer & Thompson, 2013). Although both CTLA-4 and PD-1 act as negative regulators, each modulates immune responses based on completely different scientific principles.

CTLA-4 attenuates the early activation of naïve and memory T cells by competing with CD28 for CD80 and CD84, ultimately turning off T cell activation (Linsley et al., 1996). In two large phase III clinical trials treating advanced melanoma cancer patients, ipilimumab, offered a benefit in overall survival for patients, leading to FDA approval in 2011 (Hodi et al., 2010; Robert et al., 2011). However, as it activates all T cells it leads to toxicity to normal tissues and this is the main limitation in the widespread use of anti-CTLA-4 therapy (Lynch et al., 2012; Reese, Straubhar, Pal, & Agarwal, 2015).

In normal human tissues, PD-L1 expression has only been reported on tonsil (Dong et al., 2002), placenta (Petroff, Chen, Phillips, & Hunt, 2002) and a small fraction of macrophage-like cells in lung and liver (Dong et al., 2002). In contrast, PD-L1 protein is overexpressed on a broad range of cancers

(Dong et al., 2002). PD-L1 modulates T cell activity in peripheral tissues via the interaction with PD-1 receptor on T cell. In cancer, tumour associated PD-L1 has been shown to facilitate apoptosis of activated T cells and mediate immune suppression by stimulating the production of IL-10 in human peripheral blood T cells (Dong et al., 2002). In addition, PD-L1 has been demonstrated to induce T cell dysfunction and T cell anergy (Goldberg et al., 2007; Selenko-Gebauer et al., 2003; Tsushima et al., 2007). Nivolumab and pembrolizumab are two approved anti-PD-1 mAbs. Nivolumab was approved to treat advanced melanoma and squamous NSCLC due to its significant clinical activity in advanced melanoma patients (NCT00730639, NCT01721772, NCT01844505) (Robert et al., 2015; Topalian et al., 2014) and improved overall survival in squamous NSCLC patients (NCT01642004) (Rizvi et al., 2015). Pembrolizumab was approved by FDA as a second line drug targeting metastatic melanoma. It showed similar efficacy and safety as nivolumab in a phase I trial treating advanced melanoma patients (NCT01295827) (Hamid et al., 2013; Robert et al., 2014). In a phase III clinical trial targeting advanced melanoma, pembrolizumab showed better efficacy and safety when compared to ipilimumab (NCT01866319)(Robert et al., 2015). Pembrolizumab also has shown promising effects in other cancers in clinical trials, including advanced gastric cancer (NCT01848834), head and neck cancer (NCT01848834) and classical Hodgkin's lymphoma (NCT01953692).

1.5: Cell surface glycosylation

Cell surface glycosylation is a post-translational modification of proteins and lipids, which is universal to all living cells and plays an important role in cell signalling, immune recognition and cell-cell interactions (Tuccillo et al., 2014). Monosaccharide units serve as building blocks of glycans (oligosaccharides and polysaccharides) that are synthesised by the combined action of various enzymes (i.e., glycosyltransferases and glycosidases) (Fuster & Esko, 2005; Tuccillo et al., 2014). Glycans can attach to proteins or lipids (glycoconjugates) (Figure 1.18). There are several main families of glycoconjugates: 1) the Asn-linked (N-linked) and Ser-/Thr- linked (O-linked) oligosaccharides that are present on many glycoproteins; 2) the glycosaminoglycans (GAGs) either as linear free polysaccharides (such as hyaluronan) or attached to serine residues of proteoglycans (such as heparin sulphate and chondroitin sulphate); 3) the sphingolipids which consist of oligosaccharides linked to ceramide; and 4) the glycosylphosphatidylinositol (GPI)-linked proteins, which are proteins that express a glycan chain linked to phosphatidylinositol (Fuster & Esko, 2005; Padler-Karavani, 2014).

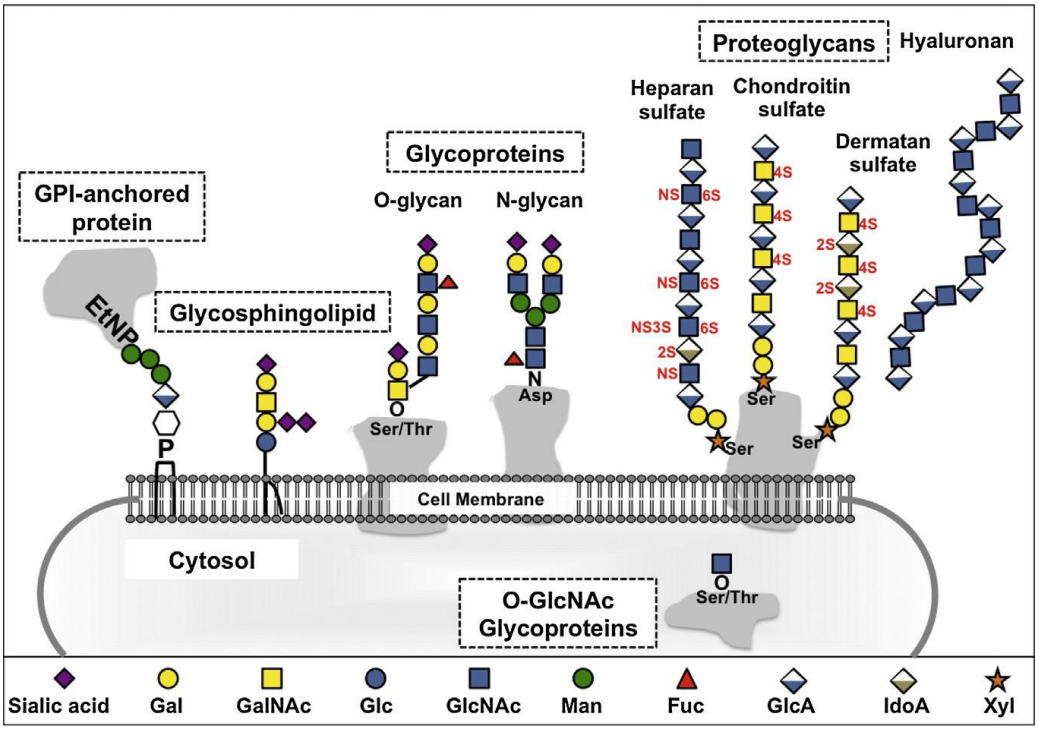


Figure 1.18: Common glycans on human cells (Padler-Karavani, 2014).

1.5.1: Glycoproteins

It has been appreciated for some time that protein glycosylation is the most complicated posttranslational modification that a protein can undergo (Spiro, 2002). More than 50% of eukaryotic proteins are glycosylated (Apweiler, Hermjakob, & Sharon, 1999). Protein glycosylation is an important process as it alters the behaviour of proteins, making them more soluble, protecting them from proteolysis, covering antigenic sites, and altering the orientation of proteins on cell surfaces (Nagae & Yamaguchi, 2012). In glycoproteins, the carbohydrate units are linked to the protein backbone by N- or O-glycosidic bonds or both, C-mannosyl bonds and phosphoglycosyl bonds and glypiated linkage (GPI-anchor) (Figure 1.19) (Spiro, 2002).

N-glycans are covalently attached to the asparagine (Asn) residues of proteins and the consensus sequence for N-glycosylation is Asn-X-Ser/Thr, where X can be any amino acid except proline. During N-linked glycosylation, proteins in the endoplasmic reticulum are decorated with a branched 9 mannose sugar $(man)_9$ complex. When the protein exits the endoplasmic reticulum, mannosidase I removes 4 of the mannose sugars $(man)_5$ and then mannosidases II removes a further 2 $(man)_3$. Glycosyltransferases then build complex glycans structures on this mannose core. Mannose is therefore never exposed on mammalian cells (Figure 1.20) (Nettleship, 2012). In O-glycosylation, the glycan is attached to the side chains of serine or threonine residues. Unlike N-linked glycosylation, no consensus sequence defining an O-linked glycosylation site has been reported (Spiro, 2002). C-

mannosylation is a novel type of protein glycosylation, which differs fundamentally from N- and O-glycosylations. It involves covalently attachment of an α-mannopyranosyl residue to the indole C2 carbon atom of tryptophan (Trp) via a C-C link (de Beer, Vliegenthart, Loffler, & Hofsteenge, 1995; Hofsteenge et al., 1994). This type of linkage was first discovered in RNase 2 from human urine (Hofsteenge et al., 1994). The phosphoglycosyl bond is another distinct type of glycopeptide linkage (GlcNac, Man, Xyl and Fuc) involving an attachment of a carbohydrate to protein via a phosphodiester bond (Haynes, 1998). Another important carbohydrate-protein connection is the GPI anchor. In this connection, Man is linked to phosphoethanolamine, which in turn is attached to the terminal carboxyl group of the protein (Ferguson, 1999).

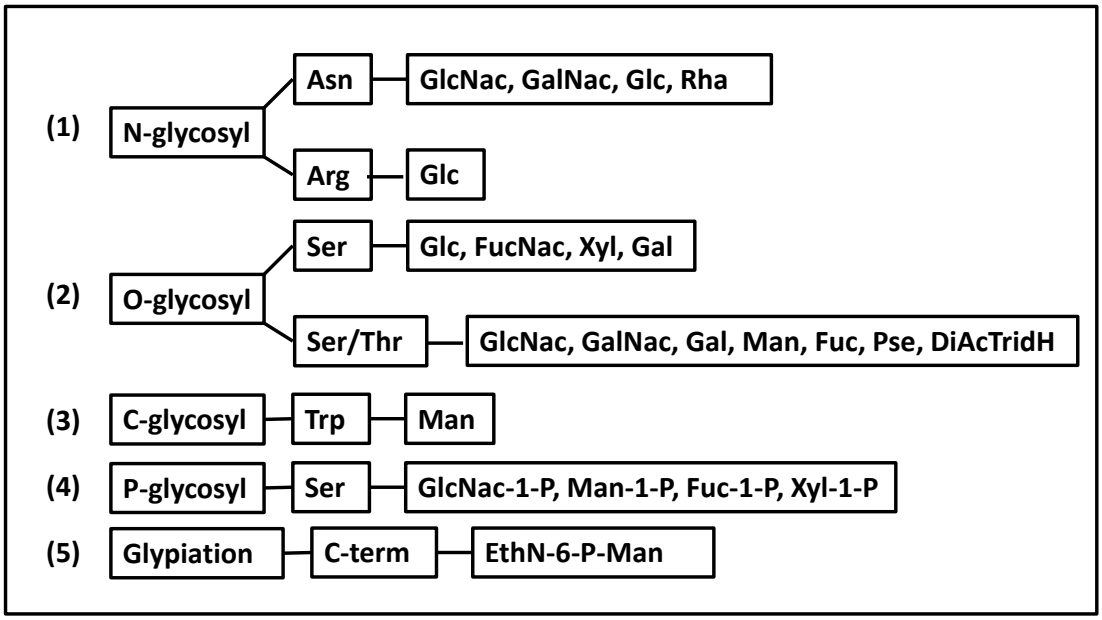


Figure 1.19: Diagrammatic representation of the five distinct types of sugar-peptide bonds. (1) N-glycosylation, (2) O-glycosylation, (3) C-glycosylation (C-mannosyl), (4) phosphoglycosylation and (5) glypiated linkage *[adapted from(Spiro, 2002)*].

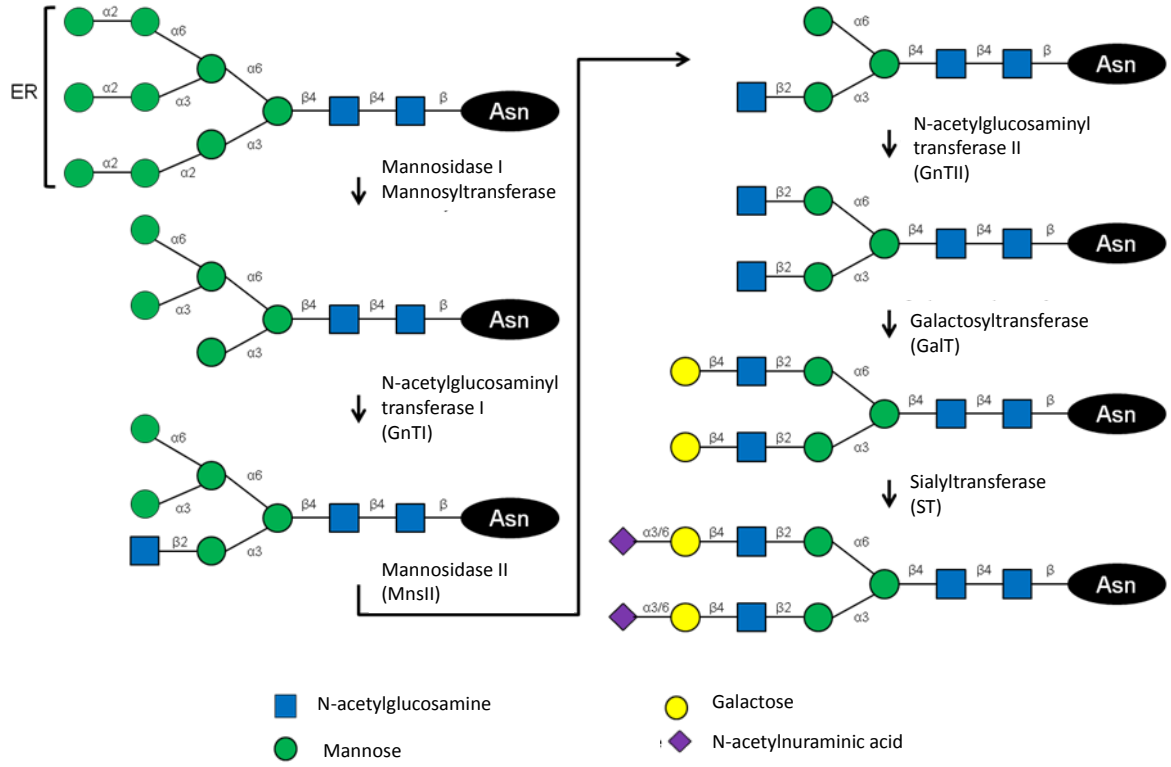


Figure 1.20: Simplified representation of the N-glycosylation pathway in humans with the initial core $Man_9GlcNac_2$ sugar being formed in the endoplasmic reticulum (ER) and further modifications taking place in the Golgi.

1.5.2: Glycolipids

Glycolipids constitute approximately 3% of the outer layer of the plasma membrane and they are composed of a lipid tail and a carbohydrate head. Glycolipids are classified into three main groups, including glycoglycerolipids, glycosylphosphatidylinositols (GPI) and glycosphingolipids (GSLs) based on the type of lipid component. Of these glycolipids, GSLs appear to be the ones that are widely overexpressed on tumours (Durrant, Noble, & Spendlove, 2012).

1.5.3: Glycosphingolipids (GSL)

In 1942, glycosphingolipids (GSLs) were first discovered and introduced by Ernst Klenk after their isolation from brain tissues. GSLs are ubiquitous membrane constituents, which are embedded in the cell plasma membrane (D. Zhang et al., 2004). 90% of mammalian GSL biosynthesis begins with the synthesis of glucosylceramide (GlcCer) which is a key precursor of the glycosphingolipid series (Lingwood, 2011). The process takes place on the cytosolic face of the Golgi complex via the action of the Type I transmembrane protein glucosylceramide (GlcCer) synthase (Ichikawa, Sakiyama, Suzuki, Hidari, & Hirabayashi, 1996), which transfers a glucose (Glu) residue to ceramide in the β-glycosidic linkage (Futerman & Pagano, 1991; Jeckel, Karrenbauer, Burger, van Meer, & Wieland, 1992). Galactose is then added to GlcCer to generate lactosylceramide (LacCer) by β-1,4-galactosyltransferases in the lumen of the Golgi apparatus (Durrant et al., 2012). Further

glycosylation steps are catalysed by different glycosyltransferases with different specificities and result in the generation of more complex GSLs (Maccioni, Giraudo, & Daniotti, 2002). The major GSL series are defined by their internal core carbohydrate sequence. They are the ganglio-series (galNacβ1-4gal), globo-series (galα1-4gal), lacto-series (galβ1-3glcNacβ1-3gal) and neolacto-series (galβ1-4glcNacβ1-3gal). LacCer provides the branch point for the synthesis of all these GSL series (Figure 1.21) (Lingwood, 2011).

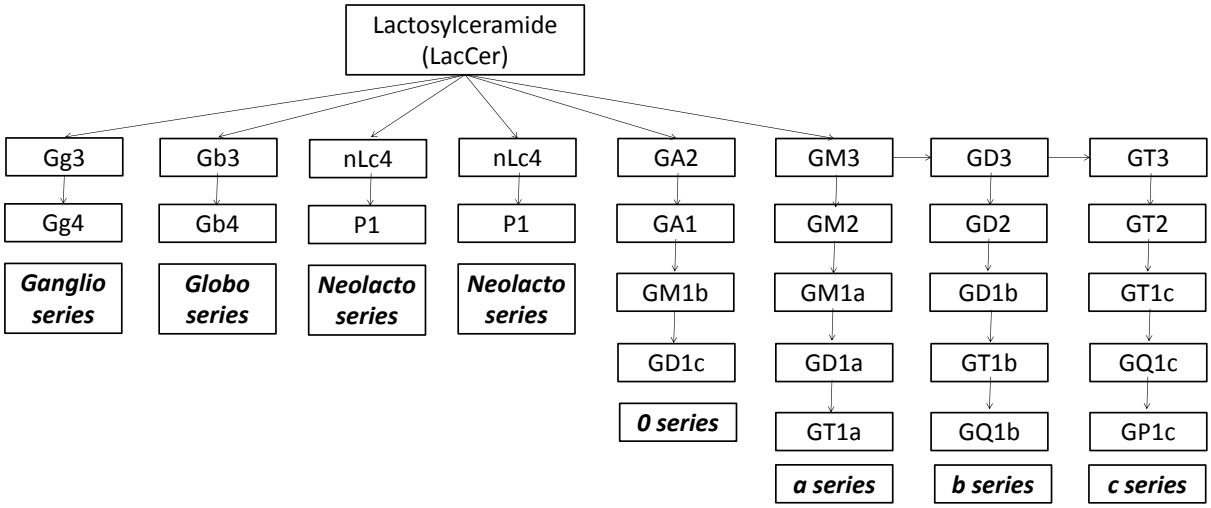


Figure 1.21: Synthetic pathways for the major GSL species. Lactosylceramide provides the branch point for different GSL series.

a) Function of GSLs

Gangliosides are expressed at different levels in different regions of the brain during brain development. Early studies on complex GSLs, particularly gangliosides, were mainly to understand their roles in the nervous system (Yates, 1986). For example, during embryogenesis, simple gangliosides GM3 and GD3 are highly expressed. However, GM3 and GD3 are reported to switch into complex gangliosides such as GD1b, GT1b and GQ1b in the adult brain tissue (Schengrund, 1990). Later on, the essential nature of GSLs for sustaining life was demonstrated in GlcCer synthase knockout mice. GlcCer synthase is the first enzyme in the GSL biosynthesis pathway. These knockout mice showed embryonic lethality (Yamashita et al., 1999).

Gangliosides on cell surface can act as toxin receptors. Two toxins, namely cholera toxin from *Vibrio cholera*, and *Escherichia coli* heat-labile enterotoxin recognise GM1 (Angstrom, Teneberg, & Karlsson, 1994) whereas Shiga toxin from *Shigella dysenteriae* recognise Gb3 (Keusch, Jacewicz, Mobassaleh, & Donohue-Rolfe, 1991). The toxins bind to GSLs where they are internalised and sent to the Golgi apparatus then to the ER, where the toxin is released and initiates toxicity (Hirst et al., 2002).

In addition, GSLs are also known as mediators of cell adhesion, for example, GSLs mediate selectin-dependent rolling and tethering (Schnaar, 2004). Importantly, carbohydrate epitopes present on GSLs can cluster and form microdomains known as 'glycosynapses', in which specific signal transducers, adhesion receptors or growth factor receptors are organised. Through glycosynapses, carbohydrate dependent adhesion creates signals that are transmitted to nucleus, leading to changes in cellular phenotype associated with cell adhesion. Glycosynapses can be divided into three types; glycosynapse I, II and III. Type I glycosynapse has been distinguished as having GSL-GSL or GSL-binding protein interaction between cells. These interactions have been described to associate with cytoplasmic signal transducers (small G-proteins, cSrc, and Src family kinases) which lead to downstream signalling and changes in transcription factor expression. Type II glycosynapses are composed of O-linked mucin-type glycoproteins which interact with carbohydrate binding protein and associate with Src family kinases. Type III glycosynapses are composed of N-linked integrin receptor complexed with tetraspanin and ganglioside (S. Hakomori, 2004).

1.6: Aberrant glycosylation in cancer

Aberrant glycosylation has been described as one of the hallmarks of cancer. Evidence is accumulating that aberrant glycosylation contributes to various aspects of cancer development and progression, including proliferation, invasion, angiogenesis, metastasis and immunity (Dube & Bertozzi, 2005; Fuster & Esko, 2005). Aberrant glycosylation in cancer is not a random process but rather is limited to a distinct subset of glycans that become modified, enriched or decreased on the tumour cell surface and mediate either promotion or inhibition of tumour progression (Dube & Bertozzi, 2005; S. Hakomori, 2002; Y. J. Kim & Varki, 1997). The two basic principles that phenotypically guide these changes are incomplete synthesis (early-stage cancers) and neo-synthesis (advanced stage cancers) (Kannagi, Izawa, Koike, Miyazaki, & Kimura, 2004) of cancer-associated cell surface glycans (S. Hakomori & Kannagi, 1983). In general, aberrant glycosylation in cancers is due to the following changes: 1) under- or overexpression of glycosyltransferases; 2) altered expression of glycoconjugate acceptor (Dennis, Laferte, Waghorne, Breitman, & Kerbel, 1987); 3) altered sugar nucleotide transporter activity (Kumamoto et al., 2001); and 4) improper function of the Golgi structure (Kellokumpu, Sormunen, & Kellokumpu, 2002) where many of the glycosyltransferases are harboured (Moremen, Tiemeyer, & Nairn, 2012).

1.6.1: Altered sialic acid expression

As early as 1960s, evidence showed that tumour cells of various origins increased the expression of sialic acids on membrane glycoproteins and glycolipids as well as their secretion into the tumour microenvironment (Forrester, Ambrose, & Macpherson, 1962; Forrester, Ambrose, & Stoker, 1964; Gasic & Gasic, 1962). Sialic acids on normal cells are involved in multiple different physiologic

processes (Bull, Stoel, den Brok, & Adema, 2014). However, hypersialylation of tumour cells specifically benefits tumour cell growth, promotes metastases (Altevogt et al., 1983; Yogeeswaran & Salk, 1981) and correlates with a poor prognosis of cancer patients (Schneider et al., 2001).

Sialic acids are nine-carbon backbone α-ketoacidic sugars (Padler-Karavani, 2014). In general, sialic acids terminate the outer end of glycans (sialoglycans) via more than 20 distinct Golgi-resident sialyltransferases (ST). This enzymatic process is carried out via their second carbon (C2) to either galactose (α2-3Gal or α2-6Gal), N-acetylgalactosamine (α2-6GalNac) or to another sialic acid (α2-8Sia) (Angata & Varki, 2002). Altogether, the different linkages to underlying sugars results in a tremendous diversity of sialoglycans (Bull et al., 2014). Sialoglycans are known to participate in cell-cell and cell-extracellular matrix interaction, including adhesion, migration and immune recognition (Bull et al., 2014). Sialic acid-binding immunoglobulin-like lectins (Siglecs) are receptor families that specifically recognise sialoglycans. Siglecs can be found on most immune cells and they can transmit immunosuppressive signals upon binding to sialic acid ligands. Thus, increased expression of siglec ligands by tumour cells could contribute to tumour immune invasion (Pillai, Netravali, Cariappa, & Mattoo, 2012).

General increase in cell surface sialic acid was shown to result from various routes (Y. J. Kim & Varki, 1997). Changes to the core structures of N-glycans are one of the most common aberrant glycosylations in cancer. Increased activity of N-acetylglucosaminyltransferase V (GlcNac-TV or MGAT5) was found to result in larger and more branched N-glycans thus providing additional acceptors for terminal sialylation (Dennis et al., 1987; Y. J. Kim & Varki, 1997). Similarly, carcinomas that overproduce mucins (heavily glycosylated high molecular weight glycoproteins; e.g. MUCI and MUC4) which contain aberrant O-linked glycosylation can lead to increased sialylation (Brockhausen, 2006; Hollingsworth & Swanson, 2004). Together with increased expression of sialyltransferases (Dall'Olio & Chiricolo, 2001), these enzymes increase cell surface sialylation and metastatic potential (Dennis et al., 1987). In addition, tumour cells often overexpress α2-6 sialic acid, mainly due to up-regulation of the ST6Gal-I (Gessner, Riedl, Quentmaier, & Kemmner, 1993; Hedlund, Ng, Varki, & Varki, 2008; P. H. Wang et al., 2003) or ST6GalNAc sialyltransferases (Julien et al., 2006; Sewell et al., 2006) that respectively conjugate terminal sialic acid to N-glycans or O-glycans and glycolipids (Dall'Olio & Chiricolo, 2001). Mass spectrometry analysis of human serum sialo-glycoproteins revealed increased expression level of α2-6 sialylation in breast cancer (Alley & Novotny, 2010) and lung cancer samples (Vasseur, Goetz, Alley, & Novotny, 2012) whereas α2-3 sialylation was increased in prostate cancer samples (Saldova, Fan, Fitzpatrick, Watson, & Rudd, 2011), malignant brain tumours (Yamamoto et al., 1997) and in ovarian serous carcinomas (P. H. Wang et al., 2005).

In 1979, Koprowoski et al. first discovered carbohydrate antigen 19-9 (CA19-9) in the SW1116 colorectal cancer cell line, by using 1116-NS-19-9 mAb (Koprowski et al., 1979). Later, CA19-9 was identified as sialyl-Lewis a antigen (SLe^a) expressed on both glycolipids and glycoproteins overexpressed on cancer cells (Kannagi, 2007). Subsequently, CA19-9 was identified in the tissue and sera of esophageal, gastric, biliary and pancreatic cancer patients (Kannagi, 2007; Koprowski et al., 1979). SLe^a has been reported to involve in cancer invasion/metastasis as it is a ligand for endothelial cell E selectin, which is responsible for cell adhesion (Duraker et al., 2007; Liao et al., 2007; Safi et al., 1987; Vestergaard et al., 1999). To date, CA19-9 is widely used as marker for predicting prognosis after pancreatic tumour resection (Berger, Meszoely, Ross, Watson, & Hoffman, 2004; Distler, Pilarsky, Kersting, & Grutzmann, 2013; M. Kobayashi et al., 2014; Mehta, Prabhu, Eshpuniyani, Kantharia, & Supe, 2010)

Aberrant expression of sialic acids confers major advantages to tumour cells. Therefore, by targeting these sialogyclans overexpressed on tumours may be highly beneficial, not only to directly limit tumour growth but also to enhance the benefit of cancer therapeutics.

1.6.2: Altered Lewis carbohydrate antigen expression

The histocompatibility blood group Lewis (Le) antigens are found on various glycoconjugates in most human epithelial tissues (Ravn & Dabelsteen, 2000). Lewis carbohydrate antigens are formed by the sequential addition of fucose onto oligosaccharide precursor chains on glycoproteins or glycolipids through the action of a set of glycosyltransferases (Figure 1.22) (Yuriev, Farrugia, Scott, & Ramsland, 2005). The Lewis antigens can be divided into 2 groups, type I (Galβ1-3GlcNac) serves as precursor for Le^a and Le^b and type II (Galβ1-4GlcNac) serves as precursor for Le^x and Le^y. Le^a and Le^b antigens are regarded as blood group antigens whereas Le^x and Le^y as tumour associated markers (Soejima & Koda, 2005). In contrast to other blood group antigens (A, B and H) which are synthesized in erythroid tissues, Le^a and Le^b are synthesized by exocrine epithelial cells, mostly of endodermal origin, secreted into plasma and secondarily acquired by erythrocyte membranes (Soejima & Koda, 2005; Yuriev et al., 2005). Le^x was reported to be overexpressed in breast and gastrointestinal carcinomas. Normal expression of Le^x is restricted on certain normal epithelial cells including the oesophagus, stomach, small bowel, ciliated epithelium of trachea, bronchus (Krug et al., 2007; Soejima & Koda, 2005) and normal human polymorphonuclear neutrophils (PMNs) (Ballare, Barrio, Portela, & Mordoh, 1995). Le^y was reported to be overexpressed on 60-90% of ovarian, breast, prostate, colon and lung carcinomas. Although Le^y expression can be found on both normal and neoplastic tissues, Le^y distribution differs between the 2 tissue types. Expression of Le^y on normal epithelial tissues is restricted to the secretory borders of epithelial surfaces, making it less accessible

to circulating antibodies. Conversely, Le^y expression on epithelial cancer cells occurs on all surfaces including luminal surfaces (Krug et al., 2007).

Sialylated Lewis carbohydrate antigens such as sialyl-Le^a and sialyl-Le^x (SLe^a and SLe^x) are significantly enhanced in cancer (Cazet, Julien, Bobowski, Burchell, & Delannoy, 2010; Kannagi et al., 2004). The expression of these cancer-associated antigens results mainly from the up-regulation of sialyltransferases (e.g. increased expression of ST3GAL IV (Cazet et al., 2010) and impairment of subsequent α2-6 GlcNac sialylation lead to expression of SLe^a, instead of the normal di-sialyl-Le^a (Tsuchida et al., 2003). Overexpression of SLe^a and SLe^x is a key event in tumour progression, invasion and metastasis (Sawada et al., 2011). SLe^a is expressed on normal fibroblasts, on the luminal side of ductal epithelial cells and some parenchymatous cells. SLe^a is normally present on the inner surface of ductal epithelium which makes it inaccessible to antibodies and immune effector cells. SLe^x can be found on granulocytes, normal oral mucosa and breast tissue (Croce et al., 2007). Both SLe^a and SLe^x are found to be aberrantly expressed on the surface of a broad range of carcinomas such as breast, ovarian, melanoma, colon, liver, lung and prostate (Heimburg-Molinaro et al., 2011).

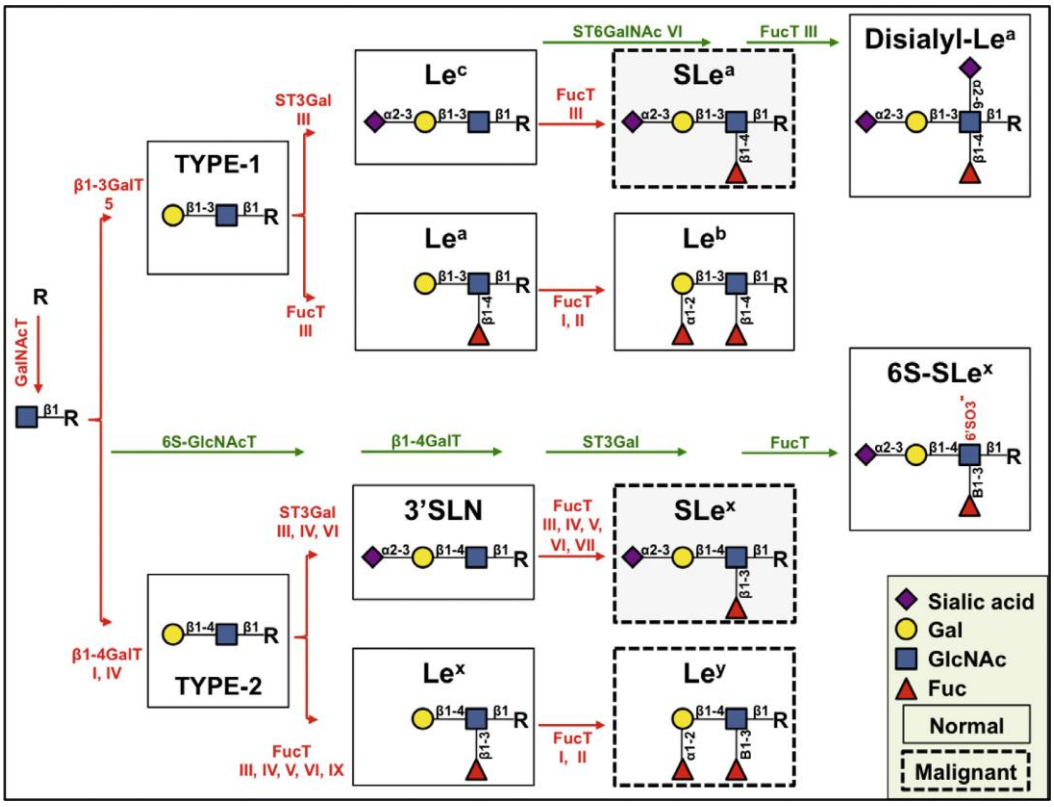


Figure 1.22: Biosynthesis of Lewis antigens. Lewis antigens are derived from the substitution of type I and type II disaccharide sequences by subsequent addition of fucose and sialic acid residues. Main enzymes involved are indicated in red and green arrows (Padler-Karavani, 2014).

1.6.3: Altered gangliosides expression

Gangliosides are acidic glycosphingolipids with the presence of at least one sialic acid linked to their oligosaccharide chain (Birkle, Zeng, Gao, Yu, & Aubry, 2003). Biosynthesis of gangliosides involves sequential addition of sialic acids to lactosylceramide (LacCer) by ST3Gal V (GM3 synthase), ST8Sial I (GD3 synthase) and ST8Sia V (GT3 synthase) that leads to the generation of the a-, b-, and c-series ganglioside precursors, respectively representing the mono-, di-, and tri-sialylated gangliosides (Figure 1.23) (Cazet et al., 2010). In general, gangliosides are involved in cell-cell recognition or regulation of downstream signalling of various proteins (e.g. insulin, epidermal growth factor, and vascular endothelial growth factor receptors) (Handa & Hakomori, 2012; Lopez & Schnaar, 2009).

Many studies indicate that tumour associated gangliosides are a result of initial oncogenic transformation and play a key role in the induction of invasion and metastasis (Birkle et al., 2003; Fredman, Hedberg, & Brezicka, 2003). Example of gangliosides that are overexpressed in cancers are: GD2 in neuroblastoma (Mujoo, Cheresh, Yang, & Reisfeld, 1987) and small cell lung cancer (SCLC) (S. C. Grant et al., 1996), GD3 in melanoma(S. Zhang et al., 1997), GM2 (Livingston et al., 2005) and fucosyl-GM1 in SCLC (S. Zhang et al., 1997).

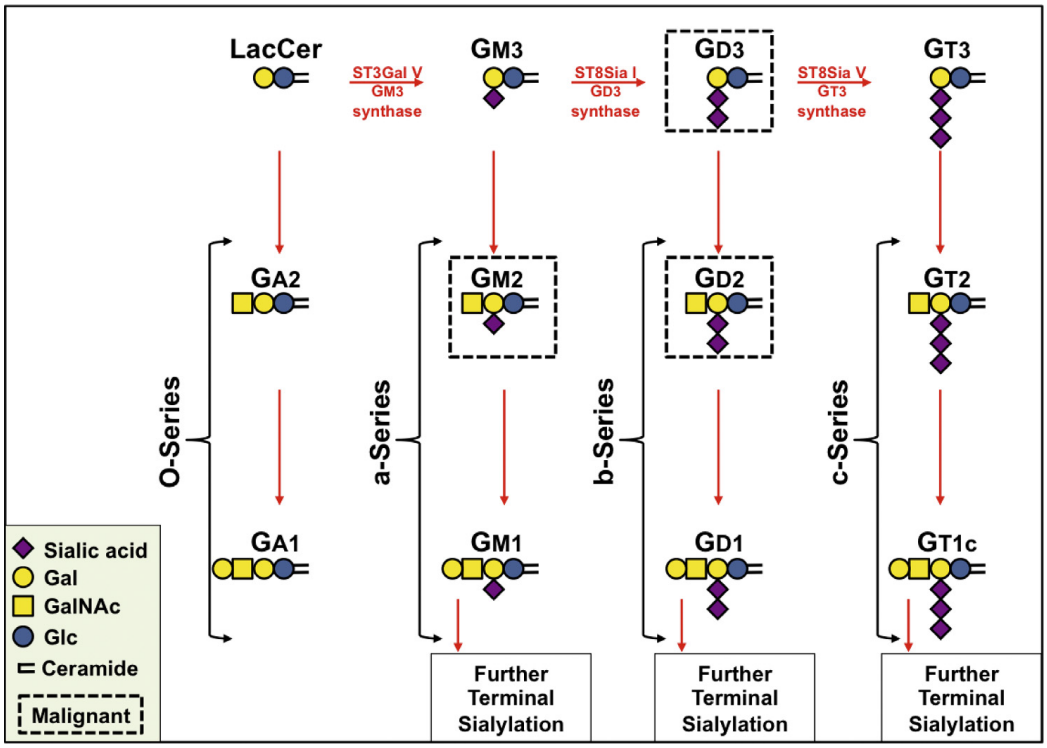


Figure 1.23: Biosynthesis of gangliosides. Lactosylceramide (LacCer) serves as the precursor for the sequential addition of sialic acids to generate the mono-, di- and tri-sialylated gangliosides. These are the precursors for biosynthesis of a-, b- and c-series gangliosides that can be further sialylated (Padler-Karavani, 2014).

1.7: Cancers

Cancer is defined by the uncontrolled growth of cells, the invasion of surrounding tissue and metastasis. Cancer is one of the leading causes of death in the modern world, with 331,487 new cases of cancer diagnosed in the UK in 2011 (CRUK, 2011). Breast, lung, prostate and bowel cancers account for over half (54%) of all new cancer cases in the UK in 2011. There were 161,823 deaths from cancer in the UK in 2012. Breast, lung, prostate and bowel cancers account for 46% of all cancer deaths in the UK in 2012 (CRUK, 2012).

1.7.1: Colorectal cancer

Colorectal cancer is the fourth leading cause of cancer-related mortality worldwide, accounting for over 600,000 deaths annually. In 2008, colorectal cancer was reported as the most common cancer (436,000 cases, 13.6% of total) and the second most common cause of death from cancer (212,000 cases, 12.3% of total) in Europe (Ferlay, Parkin, & Steliarova-Foucher, 2010).

To date, improvements in colorectal cancer treatment is attributed to early detection and diagnosis of colorectal cancer, which has the potential to prevent up to 60% of colorectal deaths (He & Efron, 2011). However, up to 56% of newly diagnosed colorectal cancer patients present with either nodal or distant metastases, with an overall 5-year survival rate of 6.6%-11.9% (Hagan, Orr, & Doyle, 2013). Therefore, further developments are necessary for the early detection and improvement of the overall quality of life of colorectal cancer patients.

Surgery, chemotherapy, radiotherapy and targeted therapies are currently the four approaches used for colorectal cancer treatment. For stage I colorectal cancer, surgical excision is usually the only treatment, as the recurrence rate for node-negative T1 colorectal cancer is rare (H. Kobayashi et al., 2011). For stage II and III colorectal patients, adjuvant therapy is the standard treatment. Stage IV patients require chemotherapy or targeted therapies combined with surgery, where appropriate.

As a consequence of improved understanding of the molecular pathology of cancer, a number of targeted agents have been developed which have demonstrated improved outcomes in metastatic colorectal cancer patients, with combination chemotherapy. Among the first of these drugs to be developed and approved for use by the Food and Drug Administration (FDA) in metastatic colorectal cancer is Bevacizumab (Hurwitz et al., 2004), Cetuximab (Van Cutsem et al., 2009) and Panitumumab (Douillard et al., 2010).

Bevacizumab is a humanised IgG1 mAb that specifically targets VEGF-A. Bevacizumab competes with VEGF from interacting VEGF receptors in vascular endothelial cells. As a result, cell signalling pathways that enhance angiogenesis, and thus the blood supply for tumours, are diminished. In

2004, Bevacizumab was approved by FDA as a first line treatment for patients with metastatic colorectal cancer. Bevacizumab is commonly used in combination with standard chemotherapeutic agents such as 5-FU as a first line treatment for patients with metastatic colorectal cancer and improves overall survival of these patients by approximately 5 months (Guan et al., 2011; Samelis, Ekmektzoglou, Tsiakou, & Konstadoulakis, 2011).

Cetuximab is a chimeric IgG1 mAb and Panitumumab is a human IgG2 mAb that specifically recognises EGFR. EGFR is a 170kDa glycoprotein located in chromosome 7 (R. L. Davies, Grosse, Kucherlapati, & Bothwell, 1980) and it is known to be overexpressed in tumours of epithelial origin, including colorectal cancer. EGFR activation is triggered by binding of EGF to the extracellular domain of the EGFR and subsequently initiates both the RAS/RAF/MAPK and the phosphatidylinositol 3-kinase (PI3K) signalling pathways. As a result, EGFR is directly involved in cell proliferation and survival, and therefore contributes to metastatic progression. Cetuximab and Panitumumab act as competitive antagonist of EGF and induce tumour cell death via ADCC and CDC (Hagan et al., 2013).

1.7.2: Breast cancer

In western countries, breast cancer is the second leading cause of cancer death among women (Rakha et al., 2008; Willems, Gauger, Henrichs, & Harbeck, 2005). In developed countries, there has been a remarkable improvement in mortality from breast cancer (Carey, 2010) which has been attributed to early detection, surgery, radiation, chemotherapy, hormone therapy and targeted therapy.

Clinically, breast cancer can be categorized into 3 distinct subtypes based on their pathophysiological features, outcomes and responses to treatments. They are the hormone receptor (HR) positive (ER and PgR), epidermal growth receptor (HER2) positive and triple negative breast cancers (TNBCs) (Santana-Davila & Perez, 2010).

HR positive breast cancers are highly associated with patient age at diagnosis, rising continuously with age (Banin Hirata et al., 2014). HR positive breast cancers can be subdivided into luminal type A [ER (+)/PgR (+)/HER2 (-)] and luminal type B [ER (+)/HER2 (+)/PgR (-)] (Santana-Davila & Perez, 2010). Luminal type A breast cancer is less aggressive whereas luminal type B is associated with increased growth rates, higher grade and overall poor prognosis. For decades, Tamoxifen has been used as first line treatment for ER-positive breast cancers. ER-positive breast cancer patients who received Tamoxifen treatment demonstrated higher survival and better prognosis (Banin Hirata et al., 2014) with few side effects (Bonneterre et al., 2000). Although endocrine therapy has revolutionized the management of breast cancer and results have improved substantially in these patients, (Banin

Hirata et al., 2014) its usefulness is limited by common intrinsic and acquired resistance (Osborne & Schiff, 2011).

HER2 is a transmembrane tyrosine kinase receptor belonging to a family of EGFRs (Banin Hirata et al., 2014). HER2 has been studied extensively in breast cancer since Slamon et al. (Slamon et al., 1987) demonstrated an association between HER2 amplification and poor prognosis (Barnes, 1993). HER2 is amplified in 20 to 30% of breast cancers (Banin Hirata et al., 2014). HER2 positive [ER (-)/PgR (-)/ HER2 (+)] breast cancer is the most chemotherapy resistant. HER2 overexpression is associated with high grade tumours, increased proliferation rates, early systemic metastasis and decreased rates of disease free and overall survival. Trastuzumab is a humanized mAb that binds to the HER2 and is used for the treatment of HER2 positive breast cancer (Slamon et al., 1987).

TNBC [ER (-)/PgR (-)/ HER2 (-)] (Santana-Davila & Perez, 2010) has a highly aggressive nature (Andre & Zielinski, 2012) and was identified as clinically important subgroup of breast cancer characterised by an especially poor prognosis. Unfortunately, after treatment of early breast cancer, TNBC patients commonly have high risk of relapse and a sharp decrease in survival in the first 3 to 5 years after treatment. The risk of distant recurrence was substantially higher in TNBC patients than those with non TNBC at approximately 3 years after diagnosis (Andre & Zielinski, 2012).

Given the majority of basal like (BL) breast cancer lack ER, PgR and HER2 expression, it has been categorized as TNBC. BL-TNBC (positive for at least one basal marker such as cytokeratin; CK5/6, CK14 or EGFR) have been described as an aggressive form of breast cancer with limited treatment options (Ho-Yen et al., 2014). BL-TNBC accounts for 8-20% of breast cancers and is more common in women harbouring BRCA1 mutations. Importantly, survival analysis shows that BL-TNBC patients do worse than women with TNBC that are negative for basal markers (Ho-Yen et al., 2014). BL-TNBC patients are of particular clinical interest because chemotherapy is the only option for systemic treatment at the moment and this patient population generally has a poor prognosis (Rakha et al., 2008).

1.7.3: Ovarian cancer

Ovarian cancer is the leading cause of death from gynaecological cancer in the Europe, with an estimated 65,697 new cases and 41,448 deaths each year (Raja, Chopra, & Ledermann, 2012). 30 out of every 100 women (30%) are diagnosed with stage 1 ovarian cancer, 4 out of every 100 women (4%) are diagnosed with stage 2, 40 out of every 100 women (40%) are diagnosed with stage 3 and 15 out of every 100 women are diagnosed with stage 4 ovarian cancer (CRUK, 2002-2006). The incidence of ovarian cancer increases with age (Kumar et al., 2010). The 5-year survival for ovarian

cancer patients with disease localised to the ovaries is >90%. However, for ovarian cancer patients with advanced disease (stage 3 and 4), their 5-year survival is <30% (Raja et al., 2012).

Serous carcinoma (80-85%) has been identified as the most common histologic type of ovarian cancer in all racial and ethnic groups, followed by endometrioid, mucinous, and clear cell carcinoma (Kurman & Shih Ie, 2010). Transitional, mixed and undifferentiated histology are the uncommon types of ovarian cancer. Symptoms of the disease are non-specific and currently there are no effective screening tests available for the general population. As a result, diagnosis often occurs when the disease is already at an advanced stage, and hence the prognosis tends to be poor (Kumar et al., 2010).

Since late 1970s, platinum-based drugs have been used as first line treatment for ovarian cancer. Treatment by cisplatin followed by carboplatin-based combinations has been the standard care for more than 15 years. The combination of cisplatin and paclitaxel was shown to be superior to cisplatin and cyclophosphamide (McGuire et al., 1996). In recent years, due to the great understanding of the molecular pathways involved in carcinogenesis, a broad range of potential therapeutic targets have been developed to block receptors, ligands or pathways. Some of them are still under development and some have been examined in first line therapy of ovarian cancer in large phase III trials (Table 6).

Table 6: First-line trials in ovarian cancer with targeted agents (Raja et al., 2012).

Target	**Agent**	**Chemotherapy**	**RCT**	**Placebo**	**C M**	**size**	**End-point**	**Trial number**
VEGF	Bevacizumab	Platinum-taxane	III	N	C+M	1500	PFS	NCT00483782 *(ICON7)
VEGF	Bevacizumab	Platinum-taxane	III	Y	C+M	2000	PFS	NCT00262847 *(GOG218)
EGFR TKI	Erlotinib	Platinum-based	III	Y	M	800	PFS	NCT00263822 (EORTC 55041/MRCOV O7)
VEGFR	Pazopanib	Platinum-based	III	Y	M	900	PFS	NCT00866697 (OVAR16)

VEGFR								
PDGFR								
FGFR	Nintedanib (BIBF1120)	Platinum-taxane	III	Y	C	1300	PFS	NCT01015118
EGFR TKI	Erlotinib	Platinum-taxane	LI	N	C+M	60	PFS	NCT00520013
VEGF	Bevacizumab						Toxicity	
VEGFR	Sorafenib	Platinum-taxane	LI	N	C+M	60	PFS	NCT00390611
PDGFR								
c-kit								
VEGFR	Sorafenib	After platinum-taxane	LI	Y	M	250	PFS	NCT00791778
PDGFR								
c-kit								
IGF R1	AMG 479	Platinum-taxane	II	Y	M	160	PFS	NCT00718523

(C: combination; OS: overall survival; M: maintenance; PFS: progression-free survival; VEGF: vascular endothelial growth factor; EGFR: epidermal growth factor receptor; VEGFR: vascular endothelial growth factor receptor; FGFR: fibroblast growth factor receptor)

1.7.4: Gastric cancer

Gastric cancer is an aggressive disease. It is the fourth most prevalent malignancy and the second leading cause of cancer-related death worldwide (Barnham et al., 1998).

To date, surgery is the only potentially curative treatment. However, most patients will ultimately recur in regional or distant sites with a 5-year survival rate of only 20-25%. Neo-adjuvant or adjuvant regional and systemic therapies are now standard care for gastric cancer patients. However, despite the integration of combined therapies, overall survival of gastric cancer patients remains low with 5-year survival rates of only 30%-36% (Cunningham et al., 2008; Macdonald et al., 2001). Chemotherapy remains to be the main treatment for gastric cancerpatients with metastatic disease,

with median survival figures of between 9 and 14 months (Cunningham et al., 2008; Wagner et al., 2006).

Agents targeting molecules such as HER-2, EGFR, VEGFRr, PI3k/Akt/mTor pathway, insulin-like growth factor receptor (IGFR), c-Met pathways, FGFR and other pathways are promising candidates for targeted therapy for GC and are now in clinical development (Table 7) (Cidon et al., 2013).

Table 7: Molecular targeted agents for gastric cancer and their targets (Cidon et al., 2013)

Target	Drugs
EGFR	Cetuximab, Panitumumab, Nimotuzumab, Matuzumab
EGFR TKI	Erlotinib, Gefitinib
HER-2	Trastuzumab
HER-2 TKI	Lapatinib
VEGFR	Bevacizumab, Ramucirumab
VEGFR TKI	Sorafenib, Sunitinib, Cediranib, Apatinib, Telatinib
PI3k-Akt-mTOR	Everolimus
FGFR	Tivantinib, Rilotumumab, Dovitinib

1.7.5: Non-small cell lung cancer

Lung cancer is one of the leading causes of cancer-related mortality worldwide. Therefore, improvement in diagnostics and treatments are needed. Lung cancer can be classified into SCLC and NSCLC. NSCLC accounts for 85% of all lung cancer cases (Navada, Lai, Schwartz, & Kalemkerian, 2006; Sher, Dy, & Adjei, 2008) and the three most common forms of NSCLC are adenocarcinoma, squamous cell carcinoma and large cell carcinoma (CRUK, 2014).

Surgical resection remains the main treatment for early-stage and localised disease (Ramalingam & Belani, 2008). However, close to 50% of lung cancer patients develop locally advanced or distant metastatic disease after curative pulmonary resection (Skrzypski et al., 2013). Combination chemotherapy is currently the standard treatment for advanced NSCLC patients with metastatic

disease (Ramalingam & Belani, 2008). Platinum-based two-drug regimens have shown to be efficacious in the first-line treatment of advanced NSCLC (Table 8) (Ramalingam & Belani, 2008).

Table 8: Comparison of two-drug combinations*, *P*= 0.029 *(Ramalingam & Belani, 2008)

Study	Regimen	Response rate	Median survival (months)	1-Year survival
n= 369	Cisplatin + etoposide	15%	9.0	37%
	Carboplatin + paclitaxel	23%	7.8	32%
ECOG 1594, n= 1155	Cisplatin + paclitaxel	21%	7.8	31%
	Cisplatin + gemcitabine	21%	8.1	36%
	Cisplatin + docetaxel	17%	7.4	31%
	Carboplatin + paclitaxel	16%	8.1	34%
TAX 326 n= 1218	Cisplatin + vinorelbine	25%	10.1	41%
	Cisplatin + docetaxel	32%	11.3	46%
	Carboplatin + docetaxel	24%	9.4	38%
SWOG 9509 n= 408	Cisplatin + vinorelbine	28%	8.1	36%
	Carboplatin + paclitaxel	24%	8.6	38%

Alteration of the major cell signalling and regulatory pathways either by overexpression or gene sequence variation is frequent event in lung cancer. The introduction of angiogenesis inhibitor [Bevacizumab; (Sandler et al., 2005)], EGFR inhibitors; Gefitinib (Kris et al., 2003) and Erlotinib (Perez-Soler et al., 2004), is changing the present and future of this disease and is certainly beneficial to lung cancer survivors (Molina, Yang, Cassivi, Schild, & Adjei, 2008).

1.7.6: Pancreatic cancer

The incidence of pancreatic cancer ranges from 1 to 10 cases per 100,000 people worldwide. Pancreatic cancer incidence is generally higher in developed countries and among men. It is the

eighth leading cause of death from cancer in men and the ninth in women throughout the world. (Ryan, Hong, & Bardeesy, 2014). Despite advancement in clinical management of pancreatic cancer, prognosis remains poor, as shown by a 1-year survival rate of approximately 18% for all stages of disease to less than 4% at 5 years (Cancer Research UK; CRUK), primarily due to the majority of tumours being at advanced stage at diagnosis (Gillen, Schuster, Meyer Zum Buschenfelde, Friess, & Kleeff, 2010).

Although surgery remains the only curative treatment for pancreatic cancer, therapeutic strategies based on initial resection have not substantially improved the survival of patients with resectable disease; presently more than 90% of patients suffer disease relapse after resection in the absence of adjuvant therapy (Hidalgo et al., 2015). Chemotherapy is the main treatment for pancreatic cancer. Gemcitabine has been used as the standard treatment for metastatic disease. Combination of gemcitabine with oxaliplatin confer a small survival benefit in younger age patients whereas combination of gemcitabine with capecitabine or other platinum analogues have not shown any improvement to patients (Berlin et al., 2002; Colucci et al., 2002; Colucci et al., 2010; Cunningham et al., 2009; Di Costanzo et al., 2005; Herrmann et al., 2005; Inal et al., 2012; Louvet et al., 2005; Poplin et al., 2009; Viret et al., 2004). Another option is a combination of chemo drugs known as FOLFIRINOX which is composed of 5-FU, leucovorin, irinotecan and oxaliplatin. In a recent phase III study, FOLFIRINOX has shown to result in longer survival compared to gemcitabine alone (11.1 versus 6.8 months respectively) as well as delaying deterioration of quality of life (Conroy et al., 2011). In addition, combination of gemcitabine with nab-paclitaxel showed improvement in overall survival compared to gemcitabine alone in a phase III Metastatic Pancreatic Adenocarcinoma Clinical Trial (MPACT) (Von Hoff et al., 2013).

A factor limiting the treatment efficacy in pancreatic cancer is impaired drug delivery due to the unique desmoplastic response (the pervasive growth of dense fibrous tissue around the tumour). To overcome this problem, several treatment strategies have been introduced, including a nanoliposomal formulation of irinotecan (MM-398); antibody drug conjugates and drugs that modify dynamic epithelial stromal interactions such as inhibitors of Hedgehog signalling (Garrido-Laguna & Hidalgo, 2015). Novel immune system checkpoint inhibitors such as mAbs targeting PD-1 or CTLA-4 (ipilimumab) have failed to show any activity in pancreatic cancer as single agents (Brahmer et al., 2012; Royal et al., 2010).

Improving the clinical outcome of pancreatic cancer patients remains an unmet need. There is a need to continue exploring early administration of chemotherapy in these patients.

Aim of Thesis

The main aim of this thesis was to generate novel mAbs targeting tumour associated carbohydrate antigens expressed on glycoproteins and glycolipids for the treatment of cancer.

Novel immunisation protocols were developed, to increase the immunogenicity of the tumour associated carbohydrate antigens.

Aims were achieved through the production of monoclonal antibodies (mAbs) recognising tumour associated carbohydrate antigens expressed on both glycoproteins and glycolipids. Characterisation of the mAbs showed promising results as potential therapeutic mAbs.

- Two closely related murine IgG3 mAbs (FG88.2 and FG88.7; FG88 mAbs) were produced from tumour plasma membrane glycolipid immunisations. FG88 mAbs recognised Lewis a/c/x glycans, which could be found on both glycolipid and glycoproteins. They were fully characterised for tumour and normal tissue binding and effector functions.
- FG88.2 mAb was chimerised and site specific mutation was used to improve the multivalent binding and direct killing activity of the chimeric mAb.
- FG2811.72 mAb (IgG3) was generated through cell immunisation. The cell used was a genetically engineered cell line, producing only specific glycolipid antigens. FG2811.72 recognised SSEA-4 glycolipid antigen and it was fully characterised for tumour and normal tissue bindings.
- The role of glycolipids and glycoproteins in inducing direct tumour cell death and mAb internalisation was investigated.

Chapter 2: Material and Methods

2.1: Tumour cell lines

Cell culture was carried out using aseptic technique in class II safety cabinet. All stock cells were obtained from liquid nitrogen storage. All cell lines were cultured in RPMI 1640, Eagle's Minimum Essential Medium (EMEM) or Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), purchased from Sigma (Poole, UK) at 37°C in 5% CO2. Cell lines were maintained by regular replacement of complete culture media and splitting to maintain log phase growth. Cell lines are summarised in Table 2.1.

Human cell line	**Source**	**Cancer type**	**Media**
Brain			
U251 MG	Gift from Dr. Steward Martin group, University of Nottingham	Glioblastoma multiforme (adult)	EMEM 10% FCS
U87 MG	ATCC (HTB-14)	Glioblastoma multiforme (adult)	EMEM 10% FCS
SF188	Gift from Dr. Steward Martin group, University of Nottingham	Glioblastoma multiforme (paediatric)	EMEM 10% FCS
KNS42	Gift from Dr. Steward Martin group, University of Nottingham	Glioblastoma multiforme (paediatric)	EMEM 10% FCS
UW2283	Gift from Dr. Steward Martin group, University of Nottingham	Medulla blastoma	EMEM 10% FCS
DAOY	ATCC (HTB-186)	Medulla blastoma	EMEM 10% FCS
Breast			
T47D	ATCC (HTB-133)	Ductal carcinoma	RPMI 10% FCS

DU4475	ATCC (HTB-123)	Breast carcinoma	RPMI 10% FCS
HCC1187	ATCC (CRL-2322)	Ductal carcinoma	RPMI 10% FCS
MCF7	ATCC (HTB-22)	Breast adenocarcinoma	RPMI 10% FCS
Colorectal			
HCT15	ATCC (CCL-225)	Colorectal adenocarcinoma (Duke C)	RPMI 10% FCS
HCT15HM2 (S. A. Watson, Morris, Crosbee, & Hardcastle, 1993)	University of Nottingham	Hepatic invasive colorectal xenograft model	RPMI 10% FCS
Colo205	ATCC (CCL-222)	Colorectal adenocarcinoma (Duke D)	RPMI 10% FCS
HT29	ATCC (HTB-38)	Colorectal adenocarcinoma	RPMI 10% FCS
Gastric			
MKN45	ATCC (CCL-171)	Gastric adenocarcinoma	RPMI 10% FCS
AGS	ATCC (CRL-1739)	Gastric adenocarcinoma	RPMI 10% FCS
Ovarian			
OVCAR3	ATCC (HTB-161)	Ovarian adenocarcinoma	RPMI 10% FCS
OVCAR5	Gift from Royal Derby Hospital	Ovarian adenocarcinoma	RPMI 10% FCS
IGROV1	Gift from Royal Derby	Ovarian adenocarcinoma	RPMI 10% FCS

	Hospital		
NSCLC			
DMS79	ATCC (CRL-2049)	Lung/pleural fluid	RPMI 10% FCS
Lymphoblast			
Jurkat	ATCC (TIB-152)	T cell leukemia	RPMI 10% FCS
Mouse cell line			
LMTK	ATCC (CCL-1.3)	Mouse fibroblast	DMEM 10% FCS
G6B3R9	Gift from IJC Josep Carreras Leukemia Research Institute	Lewis a-expressing mouse fibroblast	DMEM 10% FCS
G6B14R9	Gift from IJC Josep Carreras Leukemia Research Institute	Lewis a-expressing mouse fibroblast	DMEM 10% FCS
SSEA-3/4-LMTK	Gift from IJC Josep Carreras Leukemia Research Institute	SSEA-3/4-expressing mouse fibroblast	DMEM 10% FCS
NS0	ATCC (PA 2973)	Myeloma cells	RPMI 10% FCS

Table 2.1: Tumour cell lines used. All cell lines were regularly checked for mycoplasma contamination and authenticated using short tandem repeat (STR) profiling (by Dr Richard McIntosh and Dr Claire Seedhouse).

2.1.1: Mycoplasma check

Cell lines were checked for mycoplasma contamination using the MycoProbe Mycoplasma Detection Kit (Catalog # CUL001B), purchased from R&D systems. The hybridisation and streptavidin plates were washed with wash buffer twice. Diluted probes, positive control, negative control (sample diluent) and cell culture supernatant were added to the designated wells of the hybridisation plate. The plate was incubated for 60 min in a 65°C water bath. Then, 150µl from each well of the hybridisation plate was transferred to the streptavidin plate. The plate was incubated for 60 min on

a horizontal orbital shaker. The streptavidin plate was washed 4 times with wash buffer. Anti-digoxigenin conjugate was added to each well and incubated for 60 min on a horizontal orbital shaker. The streptavidin plate was washed 6 times with wash buffer. Substrate solution was added to each well and incubated for another 60 min on a horizontal orbital shaker. Without washing the plate, amplifier solution was added into each well and the plate was incubated on shaker for a final 30 min. Stop solution was added to each well and the optical density (O.D.) of each well was determined using a microplate reader set to 490nm.

Calculation of result: The average of the duplicate O.D. readings for each control and sample was determined. All sample average optical density values were calculated by subtracting the average negative control O.D. value. The positive control O.D. value should be ≥ 1.5. If the O.D. value is < 0.05, no mycoplasma detected; O.D. value between 0.05-0.10, result is inconclusive, test has to be repeated after 2-3 days; O.D. value > 0.10, mycoplasma detected.

Mycoplasma positive cell lines were treated with MycoKill AB (50X) (P11-016; GE Life Sciences/ PAA, Biocompare).

2.1.2: STR profiling

Genomic DNA was prepared using a commercial kit (Genepure DNA preparation kit, Invitrogen). STR PCR reactions were carried out using a commercial kit following the manufacturer's recommendations (Powerplex 16, Promega). PCR products were separated (3700 sequencer, Applied Biosystems) and results analysed using genotyping software (Applied Biosystems). STR genotyping data was checked using a commercial database (https://www.dsmz.de/).

2.2: Monoclonal antibodies

2.2.1: Commercial mAbs

CA19-9 (anti-SLe^a mAb), 225-Le (anti-Le^b mAb), P12 (anti-Le^x mAb), 7-Le (anti-Le^a mAb) and MC813 (anti-SSEA-4 mAb) were purchased from Abcam (Cambridge, UK); M1/87 from Santa Cruz Biotech (Texas, USA); CEACAM6 (anti-CEACAM6 mAb), W6/32 (anti-HLA-ABC mAb) and MC631 (anti-SSEA-3 mAb) were purchased from eBioscience (CA, USA); anti-CD40 mAb was purchased from R&D systems; anti-Fas mAb was purchased from Upstate/ Millipore (MA, USA); anti-blood group A, B and AB antibodies were purchased from Sigma (Poole, UK).

2.2.2: In house mAbs

FG88 mAbs (FG88.2 and FG88.7; anti-Le$^{a/c/x}$), FG2811.72 (anti-SSEA-4) OKT-3 (anti-CD3), FG27.18 (anti-Le^y), 791T/36 (anti-CD55) and 505/4 (anti-sialyl-di-Le$^{a/}$Le^a) mAbs were all produced in house using hybridoma technology.

2.3: Other materials

α-GalCer was purchased from Alexis Biochemical, glycans conjugated to human serum albumin (HSA) were purchased from IsoSep Ab and all lipids from Sigma.

2.4: Immunisation

2.4.1: Plasma membrane glycolipid extraction

Colo205 or SSEA3/4-LMTK cell pellets (5 x 10^7 cells) were resuspended in 500µl of Mannitol/HEPES buffer (50mM Mannitol, 5mM HEPES, pH7.2, both Sigma) and passed through 3 needles (23G, 25G, 27G) 30 times each. 5µl of 1M $CaCl_2$ was added to the cells and passed through 3 needles 30 times each as above. Sheared cells were incubated on ice for 20 min then spun at 3,000*g* for 15 min at room temperature. The supernatant was collected and spun at 48,000*g* for 30 min at 4^0C and the supernatant discarded. The pellet was resuspended in 1ml methanol followed by 1ml chloroform and incubated with rolling for 30 min at room temperature. The sample was then spun at 1,200*g* for 10 min to remove precipitated protein. The supernatant, containing plasma membrane glycolipids, was collected and stored at -20°C. (All work with chloroform and methanol was carried out in a fume hood).

2.4.2: Liposome preparation

Cancer cell line (Colo205) plasma membrane (pm) glycolipid extract (5 x 10^7 cell equiv.) was mixed with a total concentration of 10 mgs of lipids [Cholesterol, dicetylphosphate (DCP),phosphatidylcholine (PC) and α-GalCer] in a round bottom flask at various ratios (Table 2.2). The lipid mixture was then dried down using a rotary evaporator at 60°C until the solvent had evaporated, leaving a uniform lipid film on the wall of the flask. The flask was allowed to cool down to room temperature before the addition of 100µl of sterile PBS. The opening of the flask was covered with parafilm and then immersed in an ultrasonic bath for 10 min to generate liposomes. (All work with chloroform and methanol was carried out in a fume hood). For immunisation of animals with anti-CD40 mAb, the mAb (in PBS) was added to the liposome after it was made.

Table 2.2: Liposome constituents for the immunisation of one mouse.

Liposome	Cholesterol (mg)	DCP (mg)	PC (mg)	Brij97 (mg)	αGalCer (mg)	Total (mg)
5 x 10^7 cell equiv. plasma membrane lipid extract + α-GalCer	2.44	0.36	6.92	0.02	0.26	10

5 x 10^6 cell equiv. plasma membrane lipid extract + α-GalCer	2.44	0.36	6.92	0.02	0.26	10
5 x 10^7 cell equiv. plasma membrane lipid extract + anti-CD40 mAb (10µg)	2.52	0.44	7	0.02	0	10

2.4.3: Immunisation protocol

BALB/c mice were between 6 to 8 weeks old (Charles River, UK). Prior to immunisation, normal mouse serum (NMS) was collected via tail bleed extraction, and stored at -20°C. The NMS was used in latter experiments as negative controls. Mice were immunised intraperitoneally (i.p.) with either liposomes or cells at two weekly intervals using 1ml insulin syringe (BD Bioscience, Spain). Seven days after the second immunisation, and every seven days for subsequent immunisations, anti-sera was collected via tail bleed extraction and screened for IgG and IgM antibody responses. Once an animal with a high titre of IgG response was identified, it was boosted intravenously (i.v.) with liposome in the presence of α-GalCer or cells and sacrificed (by Mr. Robert Moss) 5 days later.

2.5: Generation of mAb

2.5.1: Isolation of splenocytes

Mice were sacrificed and sterilised using 70% ethanol. The spleen was collected using sterilised dissecting instruments (scissors and forceps) and transferred to sterile petri dish (100mm). The spleen was washed with 5ml serum free medium (RPMI 1640) using a 25-gauge needle and then agitated with sterile forceps gently to harvest splenocytes. 5ml of splenocytes were collected into a sterile 25ml universal tube while excess fat and connective tissues was discarded. The total fluid volume containing the splenocytes was increased to 25ml with serum free medium (RPMI 1640) and centrifuged at 100*g* for 10 min. The supernatant was removed, leaving 1ml of medium and the splenocytes. The splenocytes were then resuspended in 5ml serum free medium (RPMI 1640) and counted using a haemocytometer with trypan blue, staining for viability assessment.

2.5.2: Fusion of splenocytes with NS0 myeloma cells

Washed splenocytes were combined with healthy NS0 myeloma cells in a ratio of 1:10 (NS0: splenocytes; 1 x 10^7: 1 x 10^8 cells) in a 25ml universal tube and centrifuged at 150*g* for 5 min. The supernatant was aspirated and the combined cell pellet was resuspended in 800µl of polyethylene glycol (PEG) gently and gradually over 1 min. The cell mixture was agitated gently for 1 min prior to the addition of 1ml of serum free medium (RPMI 1640) over 1 min while continuing to agitate. A further 20ml of serum free medium (RPMI 1640) was added over 1 min while continuing to agitate. Then the cell mixture was centrifuged at 150*g* for 5 min. The supernatant was removed and the cell

mixture was resuspended in 15ml of hybridoma medium [500 ml hybridoma serum free medium (Gibco): 10ml HT (hypoxanthine thymidine) supplement (50x Hybri-Max; Sigma): 31 µl (31µg) methotrexate (1mg/ml; Sigma): 25ml of Hybridoma cloning factor (Opti-Clone II; MP): 50ml of filtered NS0 spent medium]. The cell suspension was spread evenly across a 96 well flat bottom plate and incubated at 37^{o}C in cell culture incubator (5% CO_2).

2.6: Characterisation of mAb

2.6.1: MAb isotyping

MAb was grown in hybridoma serum free medium (Invitrogen Scotland, UK) and spent medium collected. 150µl of spent medium was diluted in 1/10 dilution in PBS 1% (w/v) BSA and then pipetted into the development tube of the mouse mAb isotyping test kit (AbD Serotec, Kidlington, UK) and incubated at room temperature for 30 seconds. The tube was vortexed briefly to ensure the coloured microparticle solution was completely resuspended. One isotyping strip was placed into the tube, with the solid red end of the strip at the bottom of the tube for 5 to 10 min. The result was interpreted by checking the blue bands appeared above the letters in one of the class or subclass windows as well as either kappa or lambda window of the strip, indicating the heavy and light chain composition of the mAb.

2.6.2: MAb purification

MAb was grown in hybridoma serum free medium (Invitrogen Scotland, UK) and 2 litres of spent medium collected. 1% sodium azide (Sigma) was added to the spent medium then filtered through Whatman paper before sterilising using a 0.2µm filter (Sigma). A Protein G Sepharose column was washed with 20ml buffer C to equilibrate the column. Subsequently, the spent medium contained mAb was passed through the column overnight at 4°C. The column was washed with 50ml of buffer C to remove residual spent medium and balance the pH of the medium. 20ml of glycine was added to the column slowly and allowed to run through the column at 1ml per min. 1ml fractions were collected in 1.5ml eppendorf tubes containing 500µl of Tris-HCl. 20ml buffer C was then ran though the column to equilibrate the column. The harvested 1ml fractions were analysed using spectrophotometry with protein concentration being determined by reading optical density at 280nm, referenced against a sample of 1ml glycine, 500µl Tris-HCl. Fractions containing IgG mAb were pooled and placed into a dialysis tubing, before placing in 500ml sterile PBS and dialysed overnight at 4°C. Purified IgG mAb was quantified by spectrophotometry. All reagents for antibody purification are listed at Table 2.3.

Table 2.3: Reagents for antibody purification using Protein G sepharose column. All reagents were sterilised by passing through 0.2µl filter.	
Reagent (Sigma)	**Composition**
100mM Glycine-HCl pH 2.7	0.75g Glycine + 100ml distilled water (adjust to pH2.7 with 5M HCl)
1M Tris-HCl	0.303g Tris base + 25ml distilled water (adjust to pH9 with 5M HCl)
Na_2HPO_4 (Buffer A)	3.04g Na_2HPO_4 + 100ml distilled water
NaH_2PO_4 (Buffer B)	2.76g NaH_2PO_4 + 100ml distilled water
Buffer C	Add buffer B to buffer A until pH7.4

2.6.3: Lewis antigen (coupled to HSA), plasma membrane lipid and saliva sandwich ELISA

ELISA plates [Becton Dickinson (ie BD) Oxford, UK] were coated overnight at 4°C with 100 ng/well Lewis-HSA antigens resuspended in PBS (Isosep, Tullinge, Sweden) or 10^5 cell equivalent plasma membrane lipid (resuspended in absolute ethanol)/well or 50 ng/well FG88.2 (resuspended in PBS; sandwich ELISA), blocked with PBS/BSA and incubated with primary mAbs (direct ELISA) or 50µl/well of heat-inactivated saliva (sandwich ELISA). Antibody or Lewis antigen binding was detected using biotinylated secondary mAb (Sigma; 1/5000 dilution in PBS 1% (w/v) BSA). After incubation with Streptavidin Horseradish Peroxidase (HRPO) conjugate (Invitrogen; 1/5000 dilution in PBS 1% (w/v) BSA) and development with 3,3',5,5'-Tetramethybenzidine (TMB; Sigma), plates were read at 450nm using Tecan infinite F50. The EC_{50} for Le^a-HSA and plasma membrane lipid binding were obtained by non-linear regression of log-transformed data using GraphPad Prism 6.

2.6.4: Antibody binding to tumour cell lines

1 x 10^5 cancer cells were incubated with 50µl of primary antibodies (of various concentrations) at 4^0C for 1 hr. Cells were washed with 200µl of RPMI 10% new born calf serum (NBCS: Sigma, Poole, UK) and spun at 1000*g* for 5 min (ALC Multispeed centrifuge PK121, Jencons, UK). Supernatant was discarded and 50µl of FITC conjugated anti-mouse/anti-human or biotin-conjugated anti-mouse/anti-human IgG/IgM Fc specific antibody (Sigma; 1/100 in RPMI 10% NBCS) was used as secondary antibody. Cells were incubated at 4^0C in dark for 1 hr then washed with 200µl RPMI 10% NBCS and spun at 100*g* for 5 min. 50µl of streptavidin-FITC (Sigma; 1/100 in RPMI 10% NBCS) or streptavidin-PE-Cy7 (eBioscience; 1/100 in RPMI 10% NBCS) was used to detect biotinylated

secondary antibody. Cells were incubated at 4^0C in dark for 1 hr then washed with 200µl RPMI 10% NBCS and spun at 100g for 5 min. After discarding the supernatant, 0.4% formaldehyde (Nunc, Thermo Fisher) was used to fix the cells. Samples were analysed on a Beckman Coulter FC-500 flow cytometer (Beckman Coulter, High Wycombe, UK) or MACSQ flow cytometer (Miltenyi Biotech, Bisley, UK). To analyse and plot raw data, WinMDI 2.9 software or MACSQ software were used. Kd values for cell surface binding was obtained by non-linear regression (GraphPad Prism 6). For the competition experiments, mAbs were preincubated with a 40-fold molar excess of Le^a-HSA or Le^x-HSA (Isosep, Tullinge, Sweden) before adding to cells.

2.6.5: Binding to whole blood

50µl of healthy donor whole blood was incubated with 50µl primary antibody at 4^oC for 1 hr. The blood was washed with 150µl of RPMI 10% NBCS and spun at 100*g* for 5min. Supernatant was discarded and 50µl of FITC conjugated anti-mouse/anti-human or biotin-conjugated anti-mouse/anti-human IgG Fc specific antibody (Sigma; 1/100 in RPMI 10% NBCS) was used as secondary antibody. Cells were incubated at 4^oC in the dark for 1 hr then washed with 150µl RPMI 10% NBCS and spun at 100*g* for 5min. 50µl of streptavidin-FITC (Sigma; 1/100 in RPMI 10% NBCS) or streptavidin-PE-Cy7 (eBioscience; 1/100 in RPMI 10% NBCS) was used to detect biotinylated secondary antibody. Cells were incubated at 4^0C in dark for 1 hr then washed with 200µl RPMI 10% NBCS and spun at 100*g* for 5 min After discarding the supernatant, 50µl/well Cal-Lyse (Invitrogen, Paisley, UK) was used followed by 500µl/well distilled water to lyse red blood cells. The blood was subsequently spun at 100*g* for 5min. Supernatant was discarded and cells were resuspended in 500µl PBS. Samples were analysed on a FC-500 flow cytometer (Beckman Coulter). To analyse and plot raw data, WinMDI 2.9 software was used.

2.6.6: Erythrocyte assay

Healthy donor erythrocytes were washed 3 times in PBS and then resuspended in 10 times the packed cell volume of PBS. 50µl of washed erythrocytes were then incubated with 50µl primary antibodies at 37^0C for 1 hr. Cells were washed with 150µl of PBS and spun at 100*g* for 5 min. Supernatant was discarded and cells resuspended in 50µl FITC-conjugated anti-mouse IgG Fc-specific secondary antibody (Sigma; 1/100 dilution in PBS 1% (w/v) BSA). Cells were incubated at 37^0C in the dark for 1 hr then washed with 150µl PBS and spun at 100*g* for 5 min. Supernatant was discarded and cells were resuspended in 500µl PBS. Samples were analysed by MACSQ flow cytometer (Miltenyi Biotech, Bisley, UK) using MACSQ software.

2.6.7: Erythrocyte hemagglutination assay

4ml of normal donor whole blood was collected into a heparin tube (Becton Dickinson). The whole blood was then transferred to a sterile 15ml conical tube and washed with sterile PBS. The washed

blood was centrifuged at 100*g* for 5 min. The supernatant was aspirated. The washing step was repeated twice. After the final wash, the blood cell pellet was diluted with sterile PBS to make a final working concentration of 0.5% erythrocytes. 50µl of 0.5% erythrocytes was added into each well of a round bottom 96 well plate. On top of the erythrocytes, primary mAbs were added (50µl/well) and incubated at room temperature for 1 hr or until the erythrocytes agglutinated.

2.6.8: TLC analysis of glycolipid binding

Lipid samples were blotted onto silica plates and developed in chloroform (Sigma)/methanol (Sigma)/ distilled water (60:30:5 by volume) twice followed by hexane (Sigma): diethyl ether (Sigma): acetic acid (Sigma) (80:20:1.5 by volume) twice. The dried plates were sprayed with 0.1% polyisobutylmethacrylate (Sigma) in acetone. After air drying, the plates were blocked with PBS 2% (w/v) BSA for 1 hr at room temperature. The plates were then incubated overnight at 4°C with primary antibodies diluted in PBS 2% (w/v) BSA. The plates were then washed 3 times with PBS and incubated with biotin–conjugated anti-mouse IgG Fc specific secondary antibody (Sigma) diluted 1/1000 in PBS 2% (w/v) BSA for 1 hr at room temperature. The plates were subsequently washed again in PBS before incubating with IRDye 800CW streptavidin (LICOR Biosciences, Cambridge, UK) diluted 1/1000 in PBS 2% (w/v) BSA for 1 hr at room temperature in the dark. The plates were washed a further 3 times with PBS and air dried in the dark. Lipid bands were visualized using a LICOR Odyssey scanner.

2.7: MAb affinity

2.6.9: Affinity analysis

Surface Plasmon Resonance (SPR, Biacore X and 3000, GE Healthcare) analysis, performed by Dr Mireille Vankemmelbeke was used to investigate real-time binding kinetics of the FG88 mAbs. Polyvalent Le^a-HSA was coupled onto a CM5 biosensor chip according to the manufacturer's instructions and a reference cell was treated in a similar manner, but omitting the Le^a conjugate. A concentration range of FG88 mAbs diluted in HBS–P buffer (10 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.005% (v/v) surfactant P20) were run across the chip at a flow rate of 30 µl/min and BIAevaluation software 4.1 was used to determine the kinetic binding parameters from which affinities are calculated.

2.6.10: Glycome analysis of FG88 (Consortium for Functional Glycomics)

To determine the fine specificities of the FG88 mAbs, the antibodies were FITC labelled and sent to the Consortium for Functional Glycomics where they were screened against ≥600 natural and synthetic glycans (core H group, version 5.1). Briefly, synthetic and mammalian glycans with amino linkers were printed onto N-hydroxysuccinimide (NHS)-activated glass microscope slides, forming

amide linkages. Printed slides were incubated with 8.1 x 10^{-9} M of antibody for 1 hr at room temperature before the binding was detected with Alexa488-conjugated goat anti-mouse IgG. Slides were then dried, scanned and the screening data compared to the Consortium for Functional Glycomics database.
(http://www.functionalglycomics.org/)

2.6.11: Glycome analysis of FG2811.72 (Z Biotech, Colorado, USA)

To determine the fine specificities of the FG2811.72 mAb, FG2811.72 and MC631 (as a comparison) mAbs were sent to Z Biotech where they were screened against 15 synthetic glycans. Briefly, 15 synthetic glycans were printed on a slide and the slide incubated with FG2811.72 and MC631 mAbs at 3.6 x 10^{-7} M for 1 hr before the binding was detected with biotinylated goat anti-mouse IgG secondary antibody (7.3 x 10^{-8} M). Then the secondary antibody was detected with streptavidin-Cy3 (3.6 x 10^{-9} M). The slide was then dried, scanned and the screening data was compared to the Z Biotech glycan database.
(http://www.zbiotech.com/)

2.6.12: SDS-PAGE and Western blot analysis

10^5 or 10^6 cell equivalents (from relevant cell lines) of cell lysate, plasma membrane, total lipid extract, plasma membrane lipid extract were analysed for mAb binding. Tumour cell total and plasma membrane lipid extracts and cell lysates were reduced with dithiothreitol (DTT; Pierce Biotechnology, ThermoFisher, Loughborough, UK) and subjected to SDS-PAGE using NOVEX 4% to 12% Bis-Tris gels (Invitrogen), and transferred to PVDF membranes (Immobilon-FL, Merck-Millipore, Nottingham, UK) using 1x transfer buffer (20x, Invitrogen) and 20% (v/v) methanol at 30V for 1 hr. Membranes were blocked with 5% (w/v) non-fat dried milk powder in 0.05% (v/v) Tween-PBS for 1 hr then probed with primary antibodies diluted in Tween-PBS 2% (w/v) BSA for 1 hr at room temperature. Primary antibody binding was detected using biotin–conjugated anti-mouse IgG Fc specific secondary antibody (Sigma; 1/2000 dilution in Tween-PBS 2% (w/v) BSA) for 1 hr, and visualized using IRDye 800CW streptavidin (LICOR Biosciences, UK; 1/1000 in Tween-PBS 2% (w/v) BSA).

2.8: Immunohistochemistry assessment

To determine the therapeutic value of FG88.2 and FG2811.72 mAbs, they were screened on gastric, ovarian, small cell lung cancer, colorectal, breast (generous gift from Dr Andrew Green, Breast Cancer Pathology, University of Nottingham) and pancreatic cancer (generous gift from Dr Abid M Zaitoun, University of Nottingham) tissue microarrays (TMAs) by immunohistochemistry (IHC). They were also screened for normal cross reactivity on normal human tissue array (code number:

T8234708-2) and normal cynomolgus monkey tissue array (code number: Z7020003), both purchased from AMSBio (Abingdon, UK).

Patient cohorts: The study populations include cohorts of consecutive series of 1902 primary breast cancer (Mahmoud et al., 2011) samples (1987-1998; median follow up, 177 months (1-308 months). Patients were selected for systemic adjuvant treatment on the basis of Nottingham Prognostic Index (NPI) score and hormone receptor status [patients with NPI score ≤ 3.4 received no adjuvant therapy; those with an NPI score higher than 3.4 received tamoxifen if they were ER (+) (± goserelin if premenopausal) or classical cyclophosphamide, methotrexate and fluorouracil if they were ER (-) and fit enough to tolerate chemotherapy], 462 archived colorectal cancer (N. F. Watson et al., 2005) specimens (1994-2000; median follow up, 42 months; censored December 2003; patients with lymph node-positive disease routinely received adjuvant chemotherapy with 5-flurouracil/folinic acid), 350 ovarian cancer (Duncan et al., 2007) samples (1982-1997; median follow-up, 192 months; censored November 2005; patients with stage II to IV disease received standard adjuvant chemotherapy that in later years was platinum based), 142 gastric cancer (Abdel-Fatah et al., 2013) samples (2001-2006; median follow up, 66 months, censored Jan 2009; no chemotherapy), 68 pancreatic and 120 billary/ampullary cancer (Storr et al., 2012) samples (1993-2010; median follow up, 45 months; censored 2012; 25%-46% of patients received adjuvant chemotherapy with 5-flurouracil/folinic acid and gemcitabine), 220 non-small cell lung cancers (January 1996-July 2006; median follow up, 36 months; censored May 2013; none of the patients received chemotherapy prior to surgery but 11 patients received radiotherapy and 9 patients received at least 1 cycle of adjuvant chemotherapy post surgery) obtained from patients undergoing elective surgical resection of a histologically proven cancer at Nottingham or Derby University Hospitals (Derby, United Kingdom). No cases were excluded unless the relevant clinicopathologic material/ data were unavailable.

Methodology: Immunohistochemistry was performed using the standard avidin-biotin peroxidise method. Paraffin embedded tissue sections were placed on a 60^0C hot block to melt the paraffin. Tissue sections were deparaffinised with xylene and rehydrated through graded alcohol using Autostainer Link 48 (Dako, Cambridge, UK). The sections were then immersed in 500ml of citrate buffer (pH6) and heated for 20 min in a microwave (Whirlpool) to retrieve antigens. Endogenous peroxidase activity was blocked by incubating the tissue sections with endogenous peroxidase solution (Dako Ltd, Ely, UK) for 5 min. Normal swine serum (NSS; Vector Labs, CA, USA; 1/50 PBS) was added to each section for 20 min to block non-specific primary antibody binding. All sections were incubated with Avidin D/Biotin blocking kit (Vector Lab) for 15 min each in order to block non-

specific binding of avidin and biotin. The sections were re-blocked with NSS (1/50 PBS) for 5 min and subsequently incubated with primary antibody diluted in PBS at room temperature for an hour. Anti-β-2-microglobulin (Dako Ltd; 1/1000 in PBS) mAb and PBS alone were used as positive and negative controls respectively. Tissue sections were washed with PBS and incubated with biotinylated goat anti-mouse/rabbit immunoglobulin (Vector Labs; 1/50 in NSS) for 30 min at room temperature. Tissue sections were washed with PBS and incubated with preformed 1/50 (PBS) streptavidin-biotin/horseradish peroxidase complex (Dako Ltd) for 30 min at room temperature. 3, 3'-Diaminobenzidine tetra hydrochloride (DAB) was used as a substrate. Each section was incubated twice with 100µl of DAB solution for 5 min. Finally, sections were lightly counterstained with haematoxylin (Sigma-Aldrich, Poole Dorset, UK) before dehydrating in graded alcohols, cleaning by immersing in xylene and mounting the slides with Distyrene, plasticiser, xylene (DPX) mountant (Sigma). Staining was analysed using New Viewer Software 2010 and scored with a semiquantitative histologic score (H-score; 0-300) taking into account the staining intensity: negative (0), weak (1), moderate (2) and strong (3) and the percentage of positive cells. The stained slides were analysed by two independent observers and a consensus was agreed. Statistical analysis was performed using SPSS 13.0 (SPSS Inc.). Stratification cut off points for the survival analysis were determined using X-Tile software (Camp, Dolled-Filhart, & Rimm, 2004) and $P< 0.05$ was considered significant.

2.9: Confocal microscopy

FG88 mAbs (FG88.2 and FG88.7) were labelled with Alexa-488 fluorophore according to manufacturer's protocol (Invitrogen). 1.5 x 10^5 HCT15 cells were grown on sterile circular coverslips (22mm diameter, 0.16-0.19mm thick) in 6 well plates for 24 hr in 5% CO_2 at 37^0C. 24 hr later, cells on coverslips were treated with 3.6 x 10^{-8} M of Alexa-488-labeled FG88 mAbs for 1 hr at 37^0C in the dark before removing excess mAbs with HEPES buffer (Invitrogen). Hoechst 33258 (350/461nm) nucleic acid stain (8.1 x 10^{-9} M; Invitrogen) and LysoTracker deep red (647/668nm) lysosomal stain (50nmol/L; Invitrogen) were added during the last 30 min, with Cell Mask Orange (554/ 567nm) plasma membrane stain (1.8 x 10^{-8} M; Invitrogen) added during the final 10 min. Localisation of the mAbs was visualised using confocal microscopy through a 63 x 1.4 NA oil objective (ZEISS AX 10 Observer Z1; Carl Zeiss) with Zen 2009 image acquisition software.

2.10: ADCC and CDC

Cancer cells (2 x 10^6) were labelled with 0.925 MBq ^{51}Cr radionuclide using 200µCi sodium chromate (PerkinElmer Inc, MA) for 1 hr at 37^oC. Peripheral blood mononuclear cells (PBMCs) were isolated from a fresh human blood sample on the same day as the experiment using lymphocyte separation medium (Sigma). Human serum was collected from clotted whole blood. ^{51}Cr labelled cancer cells (5×10^3) were co-incubated with 100µl of PBMCs, 10% autologous serum or media alone, or with

mAbs at a range of concentrations [effector : target (E:T) of 100:1]. Spontaneous and maximum releases were evaluated by incubating the ^{51}Cr labeled cells with medium alone or with 10% Triton X-100, respectively. After 18 hr of incubation, 50µl of supernatant from each well was transferred to 96 well lumaplates. Plates were allow to dry overnight and counted on a Topcount NXT counter (Perkin Elmer, Cambridge, UK). The mean percentage lysis of target cells was calculated according to the following formula:

$$\text{Mean \% lysis} = 100 \times \frac{mean\ experimental\ counts - mean\ spontaneous\ counts}{mean\ maximum\ counts - mean\ spontaneous\ counts}$$

2.11: [3H] thymidine incorporation assay:

Cancer cells (1 x 10^3/well) were incubated on a 96 well flat bottom microtitre plate for 24 hr to establish an adherent monolayer. The next day, mAbs were added at 100µl/well in quadruplicate (3.3 x 10^{-11} M to 2.4 x 10^{-8} M) in the presence or absence of 20µM of a pan-caspase inhibitor Z-FMK-VAD (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone; Promega, Eastleigh, UK) for 48 hr. Cells were then exposed to 0.5µCi/well of ^{3}H-thymidine during the final 24 hr of the 48hr period. The incorporation of ^{3}H-thymidine into cell culture was measured using a liquid scintillation counter (Microscint 0 liquid scintillant on a Topcount NXT, both Perkin Elmer).

2.12: PI uptake assay

Tumour cells (5 x 10^4) were incubated on a 96 well round bottom microtitre plate with 50µl of primary antibodies at 37^{0}C for 2 hr. 1µg of PI was added and cells were further incubated at 37^{0}C for 30 min. Cells were resuspended in 500µl of sterile PBS and analysed on a FC-500 flow cytometer (Beckman Coulter). To analyse and plot raw data WinMDI 2.9 software was used. Hydrogen peroxide (1/50 in RPMI 10% NBCS) and medium alone were included as positive and negative controls respectively.

2.13: ADC assay

ADC was evaluated by measuring the cytotoxicity of immune-complexed mAbs with a saporin conjugated anti-mouse/human Fab secondary conjugated (Fab-ZAP) mouse/ human Fab/ZAP secondary conjugate [Advanced targeting system;(Kohls & Lappi, 2000)]. Cells were plated in triplicates overnight into 96 well plates (2000 cells, 90µl/well). After preincubation (30 min at room temperature) of a concentration range of mAbs with 50ng Fab-ZAP conjugate, 10µl of conjugate or free mAb was added to the wells and incubated for 72 hr at 37°C. Control wells consisted of cells incubated without conjugate, with secondary Fab-ZAP but in the absence of primary mAb and with a control mAb in the presence of Fab-ZAP. Cell viability was measured by [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (WST8) (CCK-8 kit, Sigma-Aldrich) during the final 4 hr. Plates were read at 450 nm by Tecan Infinite F50. WST8 is

reduced by dehydrogenases in live cells to generate a yellow coloured product (formazan). The amount of formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells.

2.14: DNA fragmentation

HCT15 or Jurkat cells (1.25 x 10^6) were incubated with 2.2 x 10^{-7} M of FG88.2 or FG88.7 or 3.6 x 10^{-9} M of anti-Fas (Promega) mAbs in the presence or absence of the pan-caspase inhibitor, Z-FMK-VAD (20µM final concentration), at 37^{0}C. 20 hr later, cells were collected by centrifugation at 1,400*g* for 5 min at room temperature. Cell pellets were resuspended gently in 500µl of lysis buffer (10mM Tris-HCL pH8.5, 5mM EDTA, 200mM NaCl, 0.5% SDS, all Sigma) and incubated at 60^{0}C for 5 min. RNAse was added to each sample (Sigma; final concentration of 3.2 x 10^{-8} M) and incubated at 37^{0}C for 15 min. Proteinase K was added to each sample (Active Motif, La Hulpe, Belgium; final concentration of 1.6 x 10^{-9} M) and incubated at 60^{0}C for 1 hr. 350µl of 5M NaCl was added to each sample and incubated on ice for 5 min. Samples were spun at 1,400*g* for 15 min at 4^{0}C. Supernatants were collected and an equal volume of ice cold phenol: chloroform (1:1 v/v) was added to each sample and thoroughly mixed. Samples were spun at 1,400*g* for 5 min at 4^{0}C. The aqueous phase of each sample was collected and 20µl of sodium acetate, pH 5.2 (Sigma; final concentration of 120mM) was added to each sample. 500µl of 100% ethanol (pre-chilled to -20^{0}C) was added to each sample and incubated at -80^{0}C for 1hr. Samples were spun at 1,400*g* for 10 min at 4^{0}C. Supernatants were discarded and pellets were washed with 500µl of pre-chilled 70% ethanol. Samples were spun at 1,400*g* for 10 min at 4^{0}C. Supernatants were removed, DNA pellets were allowed to air dry, resuspended in 20µl of 10mM Tris/HCl, pH8.5 buffer and analysed on a 0.8% agarose gel.

2.15: Scanning electron microscopy

HCT15 cells (1 x 10^5) were grown on sterile circular coverslips (13mm diameter, 0.2mm thick; Thermanox, Nunc, Roskilde, Denmark) in a 6 well plate at 37^{0}C, 5% CO_2. 24 hr later, cells on coverslips were treated with either 2.2 x 10^{-7} M of FG88.2 and FG88.7 mAbs, medium alone, 0.5% H_2O_2 and 0.4% saponin (Sigma) for 20 hr at 37^{0}C. 20 hr later, cells were washed with pre-warmed 0.1M sodium cacodylate buffer (pH7.4). Then the washed cells were fixed with pre-warmed glutaraldehyde (final concentration of 12.5% w/v) for 24 hr. Fixed cells were washed twice with 0.1M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide (pH 7.4) for 45 min. Subsequently, the cells were washed twice with deionised water. After the final wash, the cells were dehydrated by increasing concentration of ethanol from 40% to 100%. The prepared cells were exposed to critical point drying then sputtered with gold prior to SEM analysis (JSM-840 SEM, JEOL).

2.16: *In vivo* model

The study was conducted under a UK Home Office Licence. NCRI guidelines for the welfare and use of animals in cancer research, LASA good practice guidelines and FELAS working group on pain and distress guidelines was also followed. Endotoxin free (<10EU/ml) FG88 mAb was supplied in pre-formulated aliquots ready for dosing and stored at -20^{0}C until use. Age matched male MF-1 nude mice were obtained from Harlan Laboratories (Bichester, UK) with each group, FG88, control mAbs or the vehicle control, consisting of n≥8 animals.

Mice were implanted with HCT15HM2 DLuX cells and monitored by optical imaging to determine tumour establishment and suitability to be entered into the study. Mice were dosed with either FG88 or the positive control mAb, 505/4 (1mg/ml) at 0.1mg, 2 x weekly, 100µl intravenously (i.v.) until termination, or PBS, the vehicle control for the mAb, 100µl 2 x weekly i.v. until termination. Weekly bioluminescent imaging was carried out on all mice to obtain pre and post dosing tumour measurements. In this way each mouse provided pre-dose control readings against which tumour growth could be compared.

All measurements and readouts were transferred for data processing in SPSS v16.0. Data integrity was checked using explore and descriptive functions. Erroneous points when identified were cross referenced against the original data and corrected accordingly. The data was screened for outliers and distribution profile; data-points falling outside the 95% confidence limit (outliers) were removed from analysis, but kept in the datasheet for reference purposes.

Mice were imaged weekly for bioluminescent tumour burden (BLI) over the duration of the study as follows; 60mg/kg D-Luciferin substrate was administered subcutaneously (s.c.), the mice were anesthetised and BLI readings taken 15 min post substrate administration on open filter block (2D) and sequential emission filters (for DLIT, 3D reconstruction). Ventral and dorsal imaging was undertaken; the optimum position for imaging was abdomen uppermost. BLI was measured over the entire abdominal area, one Region of Interest (ROI) for each mouse in order to include all lesions present. Each mouse had a pre-dosing or baseline image taken to allow calculation of percentage tumour growth over time; these data were averaged per group. BLI readings were also taken after termination to identify tumours in PM tissue.

Ethics statement: Ethical approval was given to use archived human tumour samples and to produce a database by the Nottinghamshire Local Research Ethics Committee. Patient consent was not taken as the materials were all anonymised prior to use in the study. All steps have been taken to ameliorate animal suffering during this study. All works were carried out under a Home Office

License and in accordance with the Home Office Animal Welfare Guidelines. This study was approved by Nottingham University institutional review board.

Statistical analysis: Statistical analysis was performed using one or two way ANOVA followed by the Bonferroni multiple comparisons test, GraphPad Prism 6. Statistical analysis of immunohistochemical studies was performed using SPSS 13.0 (SPSS Inc.).

Chapter 3: Generation of FG88 mAbs targeting LecLx-related glycans with potent anti-tumour activity

3.1: Background

Successful antibody-mediated cancer therapy is dependent on the generation of mAbs with good specificity and potent killing. The complexity of the glycome and altered expression of glycosyl transferases associated with malignant transformation make cancer cell-associated carbohydrate excellent targets (M. N. Christiansen et al., 2014; Dalziel, Crispin, Scanlan, Zitzmann, & Dwek, 2014; Daniotti, Vilcaes, Torres Demichelis, Ruggiero, & Rodriguez-Walker, 2013; S. Hakomori, 2002). Glycolipids are particularly attractive due to their dense cell-surface distribution, mobility, and association with membrane microdomains, all of which contribute to their participation in a wide range of cellular signalling and adhesion processes (Fuster & Esko, 2005; S. Hakomori, 2002; S. I. Hakomori, 2008). Generating anti-glycolipid antibodies, however, is a challenging task as glycolipids do not provide T-cell help and the mAbs which recognise them are usually low-affinity IgMs.

To date, only a limited number of mAbs recognising glycans have been described (Durrant et al., 2012; Rabu, McIntosh, Jurasova, & Durrant, 2012). GNX-8 (human IgG1) bound the extended type I chain epitope Le^{b}-Le^{a} (M. C. Yang et al., 2009) and FC-215 (murine IgM), an anti-Le^{x} mAb that induced transient anti-tumour responses, caused profound neutropenia in phase I trials (Ballare, Portela, Schiaffi, Yomha, & Mordoh, 1998; Capurro, Ballare, Bover, Portela, & Mordoh, 1999). NCC-ST-421 (ST-421, murine IgG3) recognised gastric cancer-associated dimeric Le^{a} and demonstrated significant anti-tumour effects in a human tumour xenograft model (Watanabe et al., 1991). The murine IgM mAb 43-9F, targeted Le^{a}-Le^{x} epitopes, cross-reacting with simple and extended Le^{a} epitopes, but had no *in vivo* anti-tumour reactivity (Stroud et al., 1994). The mAb 505/4 (SC104, murine IgG1), recognised sialyl-di-Le^{a}/Le^{a}, induced ADCC, CDC as well as direct tumour cell death and more importantly demonstrated tumour growth inhibition *in vivo* (Durrant, Harding, Green, Buckberry, & Parsons, 2006). It is currently in phase I/II clinical trials in gastrointestinal cancer (NCT01447732). More recently, human SLe^{a} mAbs were produced using a patient vaccination strategy that showed specific binding to SLe^{a} and exhibited ADCC, CDC and anti-tumour activity in xenograft model (Sawada et al., 2011).

In this study, we describe the characterisation of two high affinity, IgG mAbs FG88.2 and FG88.7, which target glycans expressed by both glycolipids and glycoproteins in a wide range of tumours, but have a restricted normal tissue expression. Both mAbs displayed effective immune-mediated and direct cytotoxicity that translated into potent *in vivo* anti-tumour activity. Furthermore, their efficient internalisation suggests they could have potential as antibody drug carriers.

3.2: Results

3.2.1: Generation of murine FG88 mAbs

Three BALB/c mice (1R, 1P and 1L) were immunised by i.p. injection and boosted by i.v. injection over a period of 2 months, with plasma membrane lipid extracted from 5 x 10^7 Colo205 cells incorporated into liposomes (cholesterol, dicetylphosphate (DCP), dioleylphosphatidyl choline (DOPC) and Brij 97 to imitate the composition of the cell plasma membrane). α-GalCer and anti-CD40 mAb were used as adjuvants. The immunisation protocol is shown in Table 3.1.

Table 3.1: Immunisation schedule. (pm: plasma membrane; α-GalCer: alpha-galactosylceramide)

	Mouse 1R	Mouse 1L	Mouse 1P
1st immunisation	Colo205 pm lipid extract (5 x 10^7) + α-GalCer	Colo205 pm lipid extract (5 x 10^7) + α-GalCer	Colo205 pm lipid extract (5 x 10^7) + α-GalCer
2nd immunisation	Colo205 pm lipid extract (5 x 10^7) + anti-CD40	Colo205 pm lipid extract (5 x 10^7) + anti-CD40	Colo205 pm lipid extract (5 x 10^7) + anti-CD40
3rd immunisation	Colo205 pm lipid extract (5 x 10^7) + α-GalCer	Colo205 pm lipid extract (5 x 10^7) + α-GalCer	Colo205 pm lipid extract (5 x 10^7) + α-GalCer
Boost	Colo205 pm lipid extract (5 x 10^6) + α-GalCer	-	-
Fusion	FG88.2 and FG88.7	-	-

To assess whether the immunised mice (1R, 1L and 1P) generated anti-glycolipid antibody responses, mouse anti-sera were screened against Colo205 total lipid extracts by lipid enzyme-linked immunosorbent assay (ELISA) (Figure 3.1). Serum dilutions used were 1/500, 1/1,000, 1/5,000 and 1/10,000. By 6 weeks post two immunisations, all three mice demonstrated high Colo205 lipid-antibody binding titre with mouse 1R showing the highest antibody titre (end point titre >1/10,000) followed by 1L and 1P (end point titre 1/5,000). At the 1/500 dilution, mouse 1R serum demonstrated the highest binding to Colo205 lipids with optical densities (OD) of 0.88 followed by mouse 1P (0.62 OD units) and mouse 1L (0.55 OD units). 505/4 mAb which is a mAb recognising sialyl-di-$Le^{a/}Le^{a}$, was included as positive control (0.96 OD units). Normal mouse serum (untreated) (0.06 OD units) and PBS (0.06 OD units) were used as negative controls.

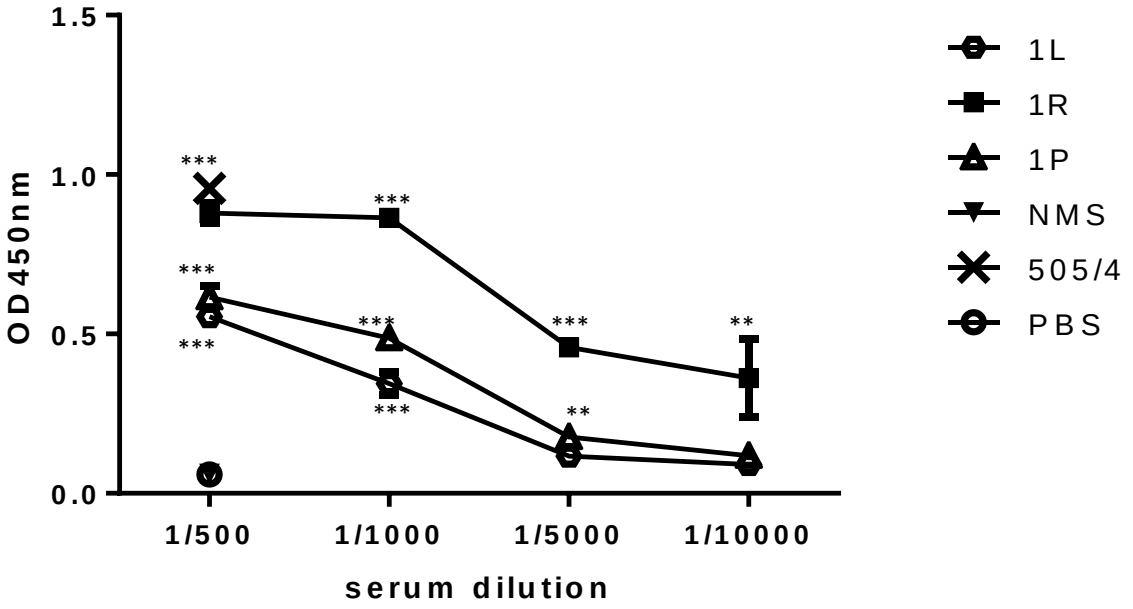


Figure 3.1: ELISA titration of immunised mice anti-sera (1R, 1L and 1P) against Colo205 total lipid extract. Colo205 total lipid extract was dried onto a 96 well flexi-plates. Plates were blocked with PBS 2% BSA before incubating with serial dilutions of mice anti-sera and probed with biotinylated anti-mouse IgG (Fc specific) secondary antibody followed by detection with streptavidin-HRPO. 505/4 mAb was included as positive control whereas normal mouse serum (NMS) and PBS were used as negative controls. Error bars represent standard deviation of quadruplicate wells. Significance between mice anti-sera and PBS control was established by one way ANOVA (*, P< 0.05; **, P< 0.01; ***, P< 0.001 versus control).

To confirm that the mouse anti-sera recognised glycolipid antigens, Colo205 total lipid extract and plasma membrane lipid extract were subjected to dot blot TLC and immunostaining with mice anti-sera (1L, 1R and 1P) at a 1/500 dilution. Phosphatidylethanolamine (PE), phosphocholine (PC) and cholesterol were included as negative controls. 505/4 mAb and NMS were used as positive and negative antibody controls respectively (Figure 3.2). As shown in Figure 3.2, all three mouse anti-sera and 505/4 mAb stained glycolipids on Colo205 total lipid extract and plasma membrane lipid extract but not PE, PC and cholesterol. NMS did not show any staining on glycolipids.

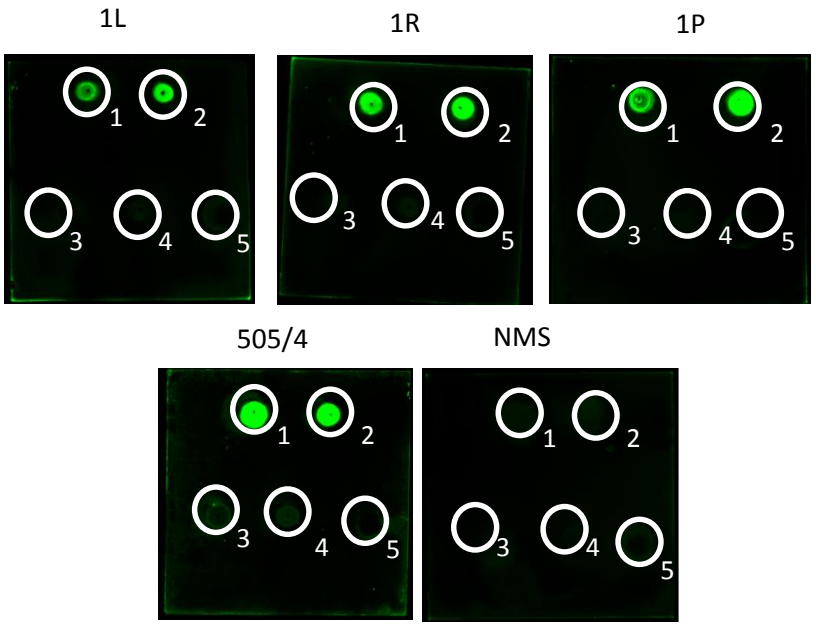


Figure 3.2: Binding of mice anti-sera (1R, 1L and 1P) to Colo205 glycolipid antigens were assessed by dot blot thin layer chromatography (TLC) analysis. Dot blot thin layer chromatography analysis of 1) Colo205 plasma membrane lipid extract (4 x 10^6 cells equiv.), 2) Colo205 total lipid extract (1 x 10^6 cells equiv.), 3) phosphatidylethanolamine (PE), 4) phosphocholine (PC) and 5) cholesterol using mouse anti-sera at 1/500 dilution. 505/4 mAb (3.6 x 10^{-8} M) and NMS (1/500) were included as positive and negative control respectively.

PAGE gel electrophoresis and Western blot analyses of Colo205 cell lysates, total lipid extract and plasma membrane lipid extract using mice anti-sera (1R, 1L and 1P) at 1/1,000 dilution demonstrated that mouse 1R recognised two low molecular weight proteins (25-30 kDa) as well as a band at the dye front, which is presumed to be glycolipid (Figure 3.3). Mouse 1P showed similar level of protein recognition but only low level glycolipid binding. In contrast, mouse IL only showed protein recognition but not glycolipid. Only serum from mouse 1R had detectable antibody which bound glycolipid antigens. The positive control mAb, 505/4, recognised low, intermediate and high molecular weight proteins as well as the dye front band. No antibody reactivity was seen with NMS.

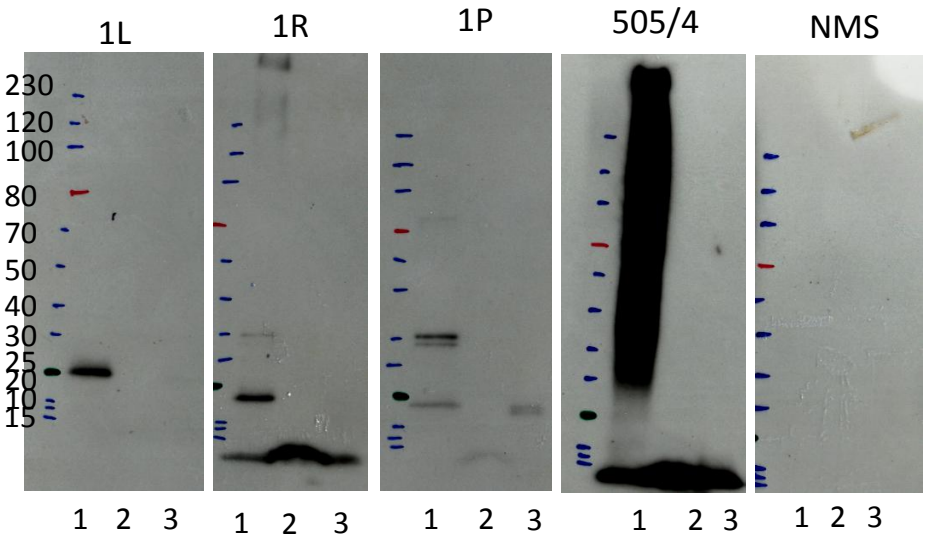


Figure 3.3: Binding of immunised mouse anti-sera (1L, 1R and 1P) to protein and lipid antigens were assessed by Western blot analysis. PAGE gel electrophoresis and Western blot analysis of 1) Colo205 cell lysate (1 x 10^5 cells equiv.), 2) Colo205 total lipid extract (1 x 10^6 cells equiv.) and 3) Colo205 plasma membrane lipid extract (4 x 10^6 cells equiv.) using immunised mouse anti-sera (1L, 1R and 1P) at 1/1,000 dilution. 505/4 (3.6 x 10^{-8} M) mAb and NMS (1/1,000) were included as positive and negative controls respectively.

Next, binding of all three mouse anti-sera to Colo205 cells was shown by indirect immunofluorescence and flow cytometric analysis (Figure 3.4). All three mouse anti-sera showed strong binding intensity to Colo205 cell surface antigens at 1/500 dilution with mouse 1R the strongest binder [1L Geometric mean (Gm): 676; 1R Gm: 1627; 1P Gm: 1059]. 505/4 (Gm: 6430) and W6/32 (Gm: 2821) mAbs were used as positive controls whereas NMS (Gm: 18), IgG isotype control (IgG ctrl; Gm: 14) and medium (RPMI; Gm: 14) were negative controls.

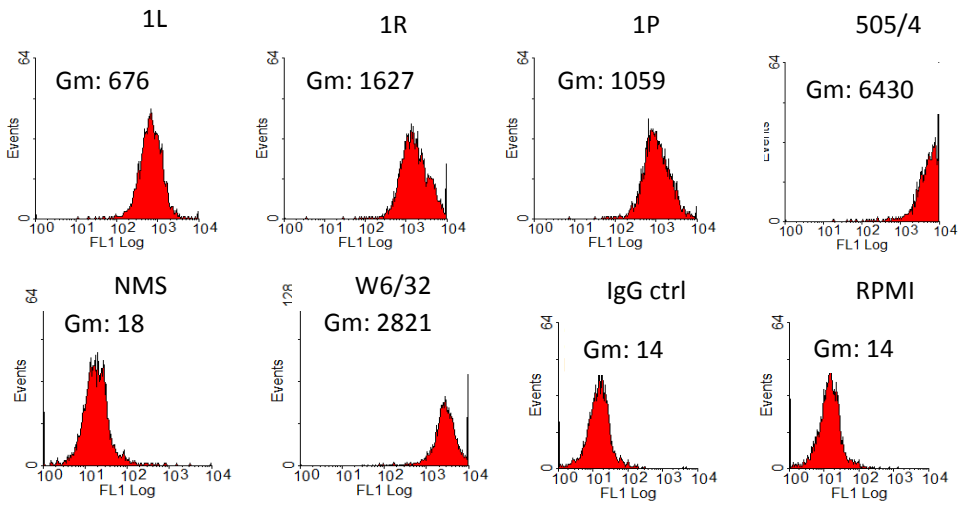


Figure 3.4: Binding of mouse anti-sera to Colo205 cell surface antigens. Binding of mouse anti-sera to Colo205 cells was assessed by indirect immunofluorescent staining and flow cytometric analysis. In these assays, 505/4 (3.6 x 10^{-8} M) mAb and W6/32 (3.6 x 10^{-8} M) antibody (anti-human class I histocompatibility antigens (HLA-A,B,C) were included as positive controls whereas PBS, IgG isotype control antibody and NMS were included as negative controls.

Due to the positive response to Colo205 cell surface glycolipid antigens, mouse 1R which also showed the strongest antibody response was boosted and sacrificed and mAbs FG88.2 and FG88.7 were generated by fusing splenocytes from mouse1R with the mouse myeloma cell line, NS0.

3.2.2: Characterisation of FG88 mAbs (FG88.2 and FG88.7)

3.2.2.1: DNA sequence

DNA sequencing revealed that FG88.2 (Figure 3.5a and b) mAbs belonged to the IGHV6-6*01 heavy chain and IGKV12-41*01 gene families where it has ten mutations from IGHV6-6*01 (Figure 3.5c) and eleven mutations from IGKV12-41*01 (Figure 3.5d). FG88.7 has eight mutations from IGHV6-6*01 and twelve mutations from IGKV12-41*01 (Figure 3.6a and b). The nature and pattern of mutations suggests affinity maturation. The two FG88 mAbs differ by only three residues, two in the CDRs and one in the FR region, suggesting that they would recognise very closely related antigens.

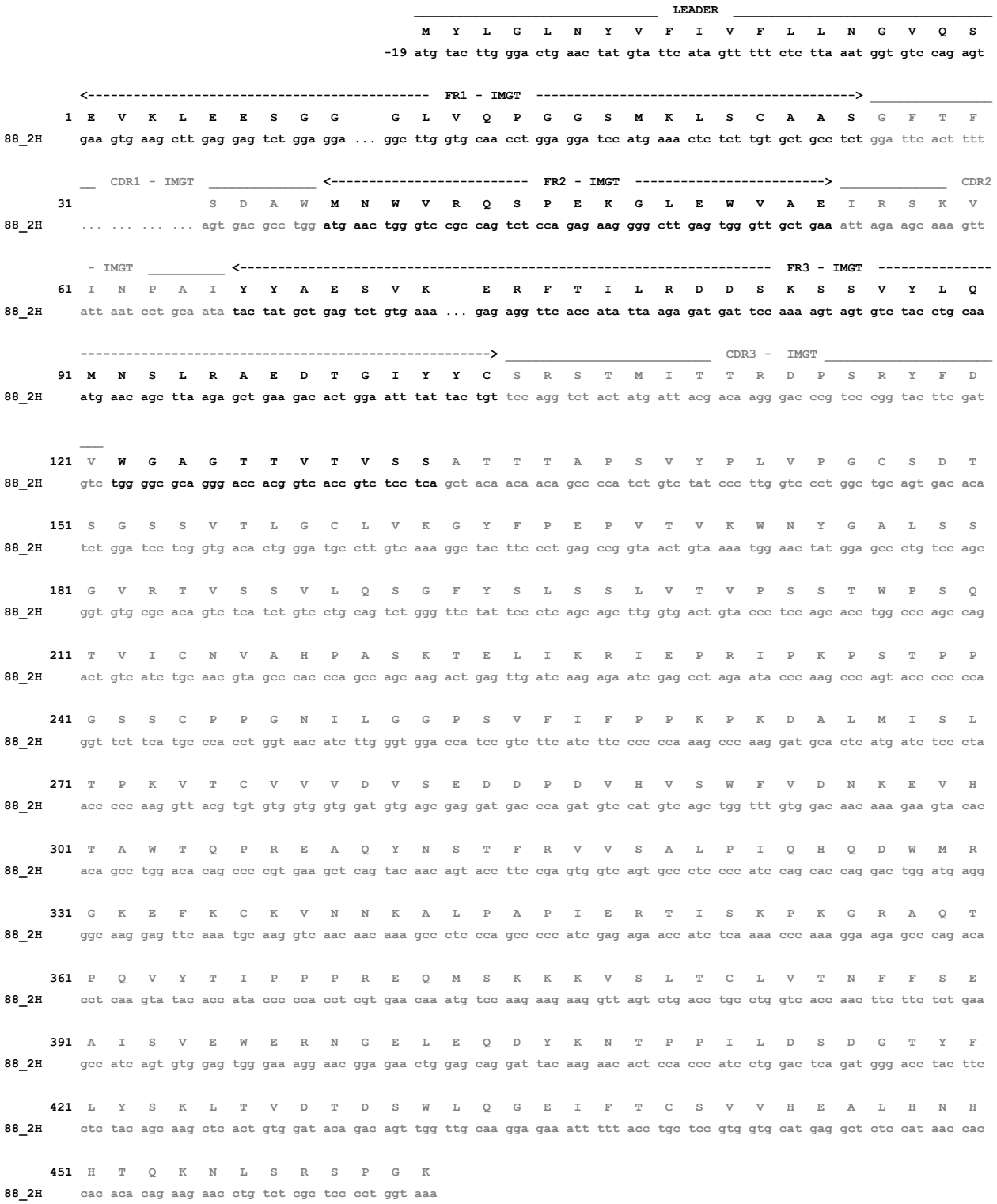


Figure 3.5a: Amino acid and nucleotide sequence of FG88.2 IgG3 heavy chain. Nucleotide and amino acid sequence of the FG88.2 heavy chain, showing the antibody leader sequence (removed from the mature antibody), framework regions (FR) 1 to 3 and complementarity determining regions (CDR) 1 to 3. The antibody constant region (following CDR3) is shown in a lighter coloured font.

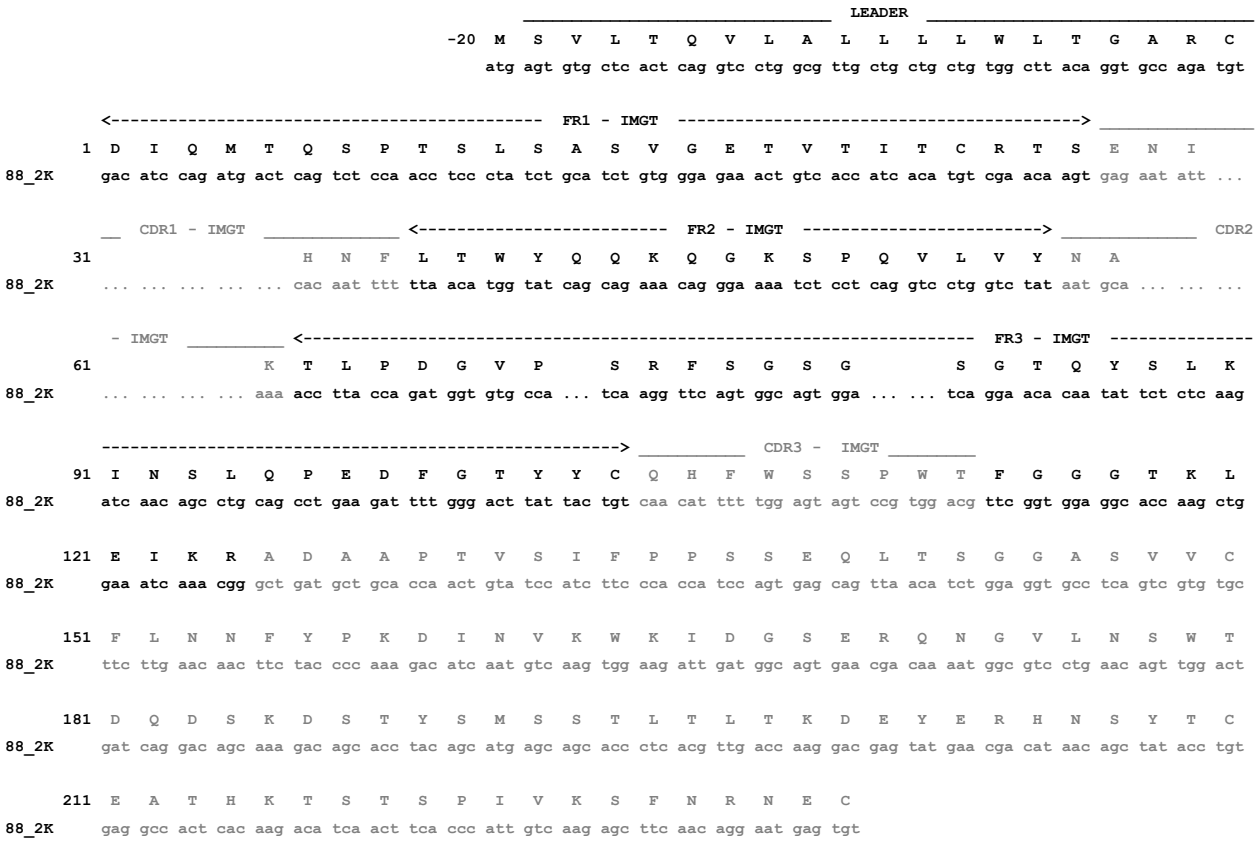


Figure 3.5b: Amino acid and nucleotide sequence of FG88.2 mouse kappa light chain. Nucleotide and amino acid sequence of the FG88.2 kappa chain, showing the antibody leader sequence (removed from the mature antibody), framework regions (FR) 1 to 3 and complementarity determining regions (CDR) 1 to 3. The antibody constant region (following CDR3) is shown in a lighter coloured font.

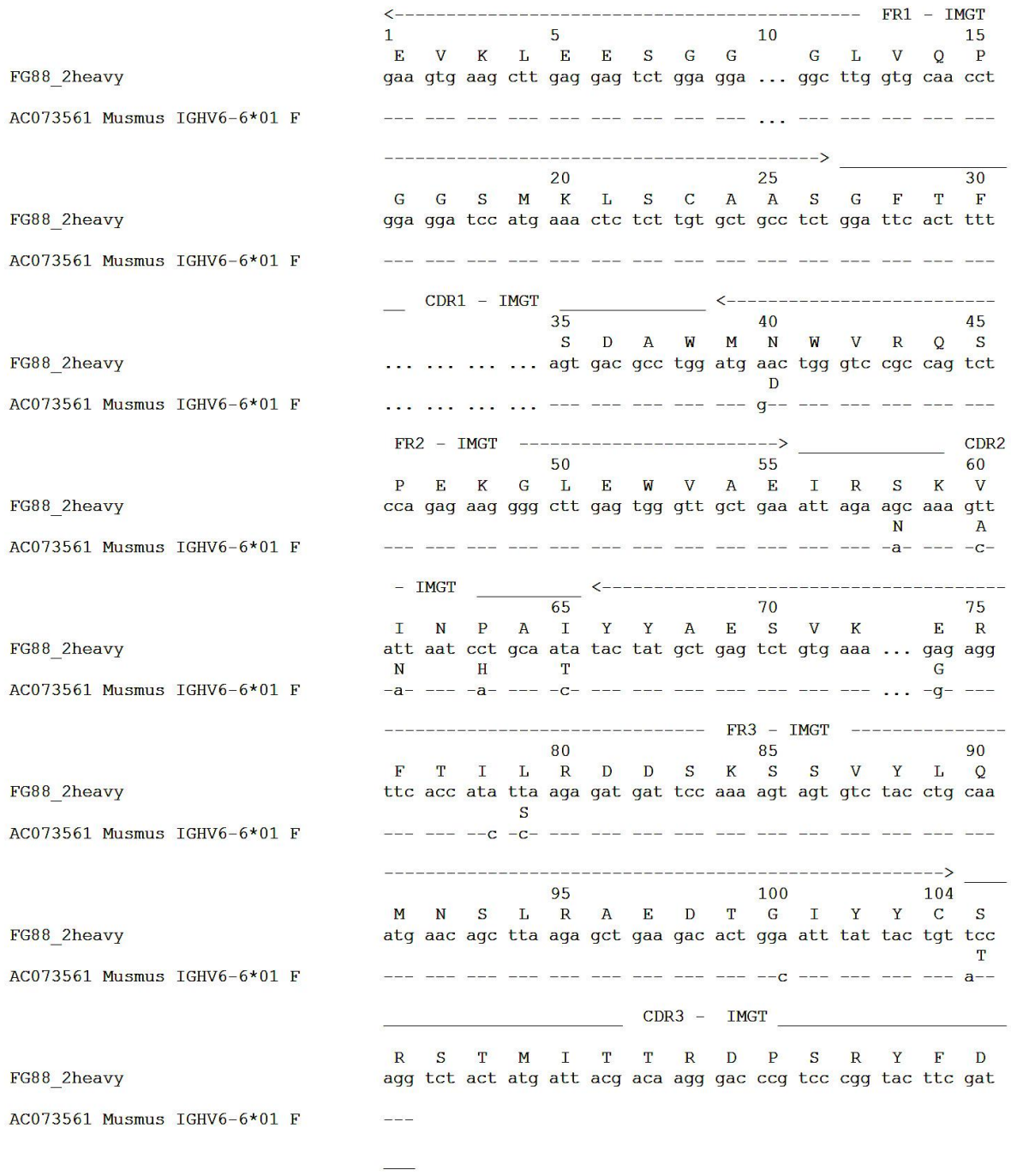


Figure 3.5c: Amino acid and nucleotide sequence of FG88.2 IgG3 heavy chain compared to germline V region IGHV6-6*01. Nucleotide and amino acid sequence of the FG88.2 heavy chain, showing the framework regions (FR) 1 to 3 and complementarity determining regions (CDR) 1 to 3.

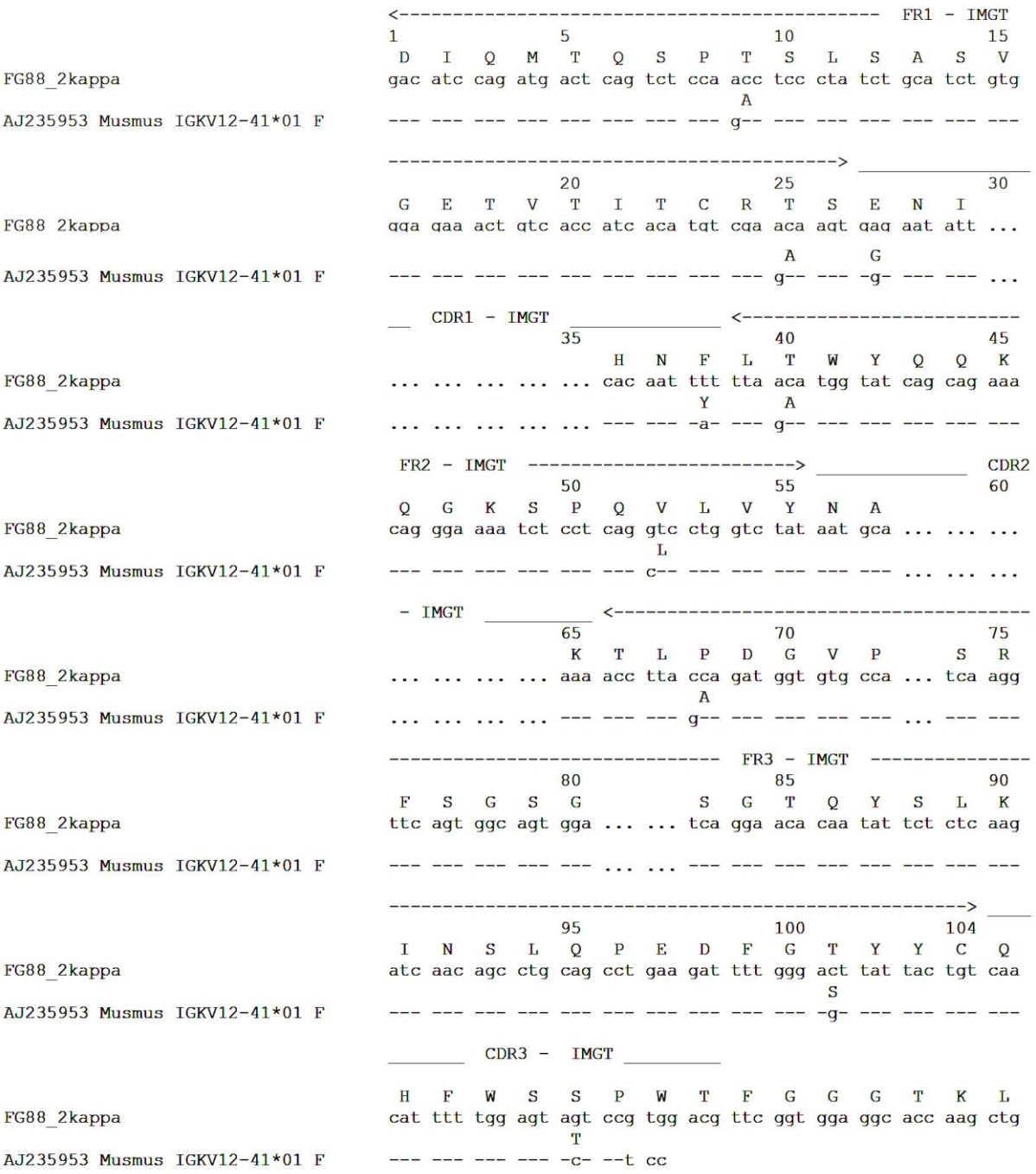


Figure 3.5d: Amino acid and nucleotide sequence of FG88.2 kappa chain compared to germline V region IGKV12-41*01. Nucleotide and amino acid sequence of the FG88.2 kappa chain, showing the framework regions (FR) 1 to 3 and complementarity determining regions (CDR) 1 to 3.

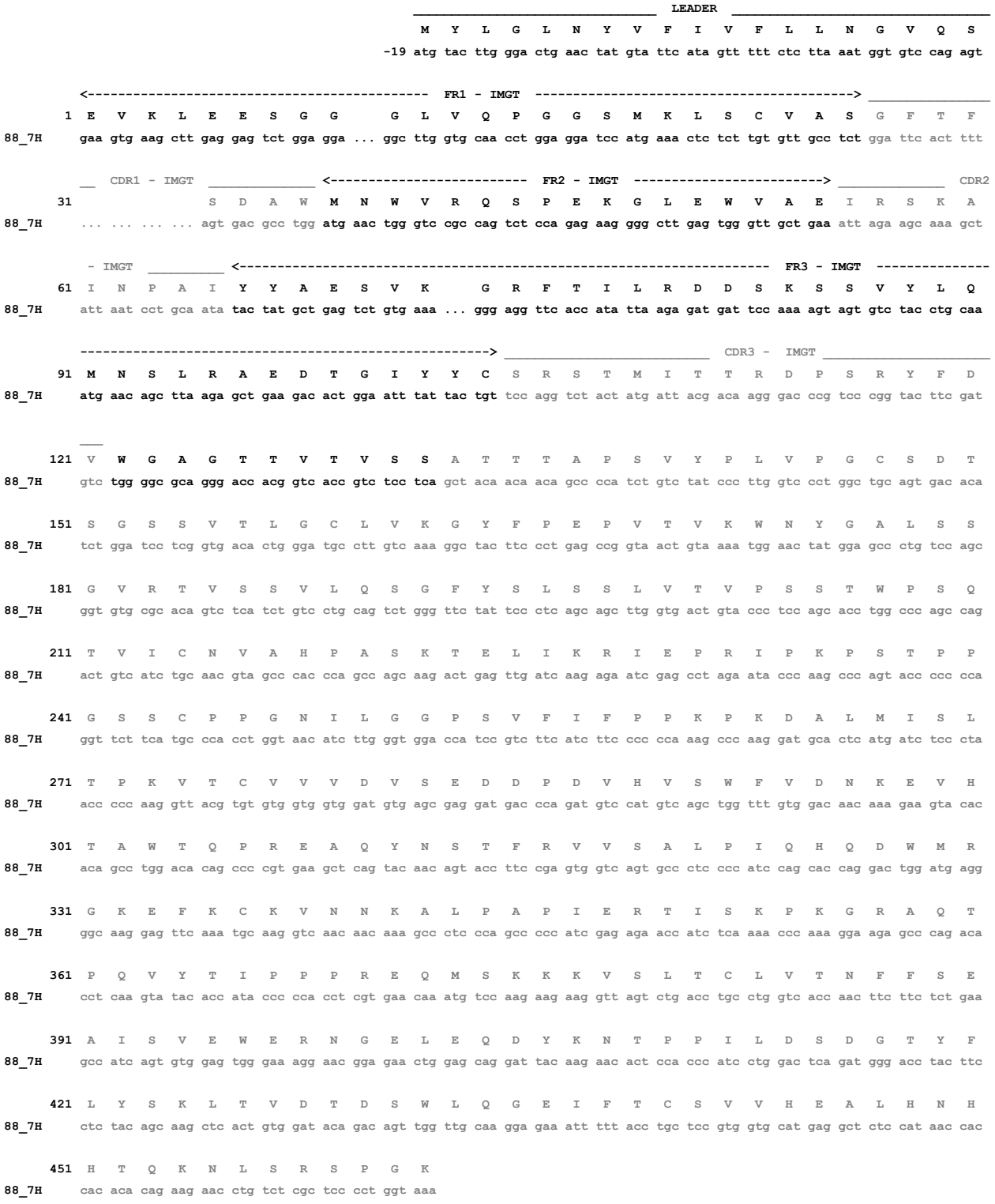


Figure 3.6a: Amino acid and nucleotide sequence of FG88.7 IgG3 heavy chain. Nucleotide and amino acid sequence of the FG88.7 heavy chain, showing the antibody leader sequence (removed from the mature antibody), framework regions (FR) 1 to 3 and complementarity determining regions (CDR) 1 to 3. The antibody constant region (following CDR3) is shown in a lighter coloured font.

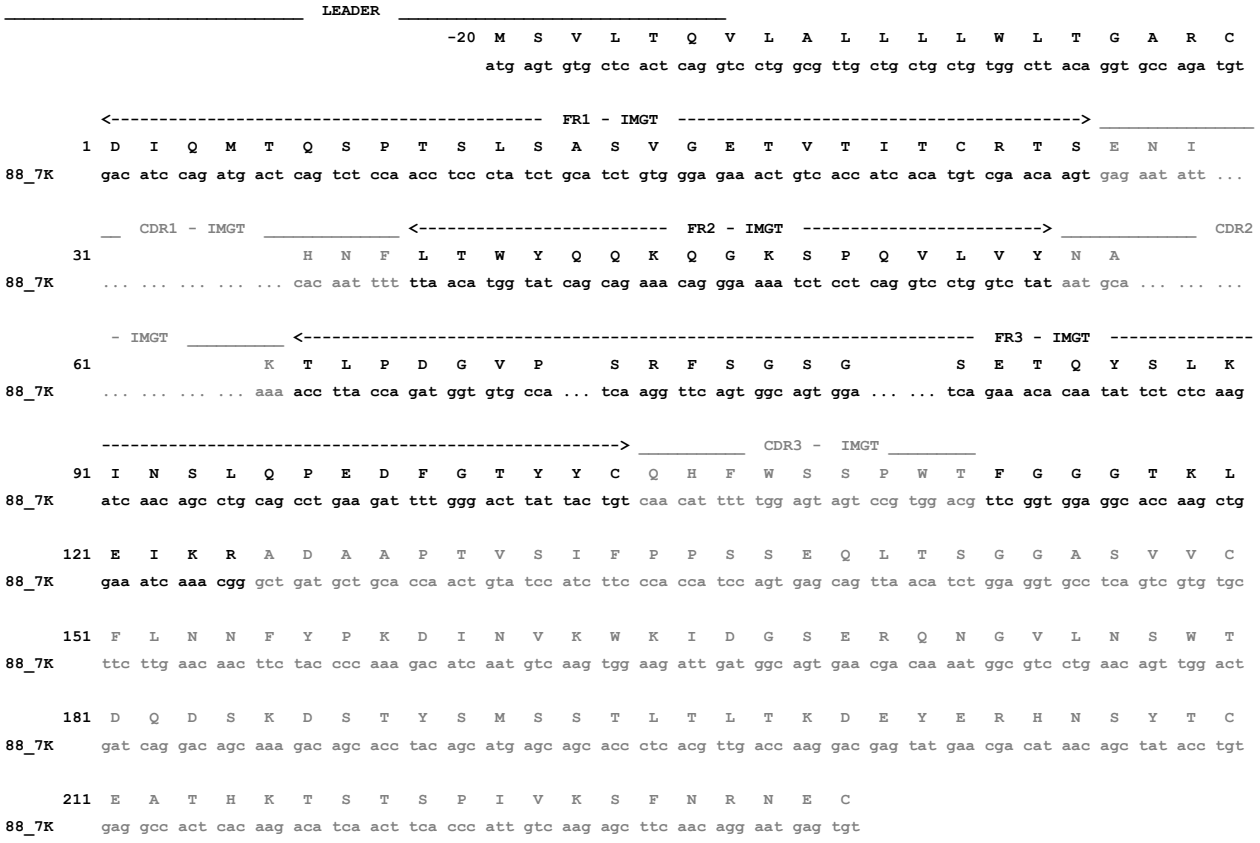


Figure 3.6b: Amino acid and nucleotide sequence of FG88.7 mouse kappa light chain. Nucleotide and amino acid sequence of the FG88.7 kappa chain, showing the antibody leader sequence (removed from the mature antibody), framework regions (FR) 1 to 3 and complementarity determining regions (CDR) 1 to 3. The antibody constant region (following CDR3) is shown in a lighter coloured font.

3.2.2.2: Glycoepitope characterisation

Both FG88 mAbs were mouse IgG3 isotypes with κ light chains. The purified FG88 mAbs showed strong Colo205 cell-surface reactivity by flow cytometry analysis (FG88.2 Gm: 4538; FG88.7 Gm: 2897) when compared to positive control mAb 505/4 recognising sialyl-di-Le^{a}/Le^{a}, (Gm: 8694) and negative control of mouse IgG isotype control (IgG ctrl; Gm: 25) (Figure 3.5) and they recognised glycoepitopes on glycolipids and glycoproteins (MW ranging from 10 to > 230kDa) in lipid extracts and cell lysates from Colo205 by Western blot analysis (Figure 3.6).

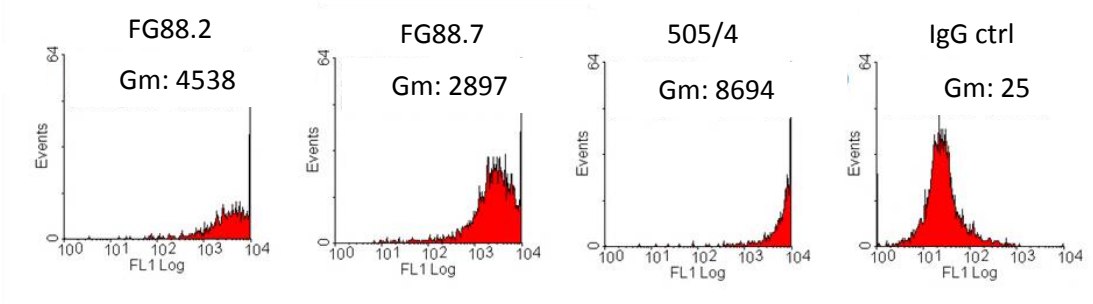


Figure 3.5: Binding of mAbs to Colo205 cell surface antigens. Binding of FG88.2 (3.6 x 10^{-8} M) and FG88.7 (3.6 x 10^{-8} M) to Colo205 cells were assessed by indirect immunofluorescence staining and flow cytometric analysis. Binding of these mAbs were compared to the positive control, 505/4 (3.6 x 10^{-8} M); and the negative control mouse IgG isotype control (3.6 x 10^{-8} M). Results are expressed as Gm values.

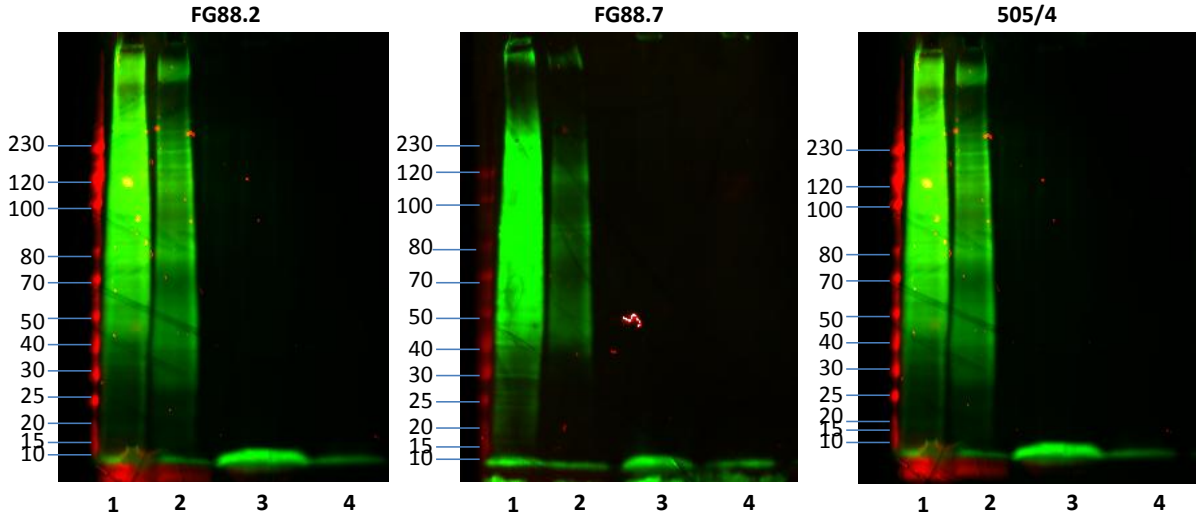


Figure 3.6 Binding of FG88.2 and FG88.7 mAbs to protein and lipid antigens were assessed by Western blot analysis. PAGE gel electrophoresis and Western blot analysis of 1) Colo205 cell lysates (1x10^{5} cells equiv.), 2) Colo205 plasma membrane (1x10^{6} cells equiv.), 3) Colo205 total lipid extract (1x10^{6} cells equiv.) and 4) Colo205 plasma membrane lipid extract (1x10^{6} cells equiv.) using FG88.2 mAb, FG88.7 mAb and 505/4 mAb all at 3.6 x 10^{-8} M.

Confirmation and further characterisation of glycolipid reactivity came from HPTLC analysis of a panel of tumour cell total lipid extracts and immunostaining with FG88 mAbs. 505/4 (anti-sialyl-di-Le^{a}/Le^{a}) and CA19-9 mAbs (anti-sialyl-Le^{a}) were included in the same panel for comparison (Figure 3.7). As shown in Figure 3.7, all 4 mAbs stained glycolipids expressed on Colo205 (colorectal line), MKN45 (gastric line) and HCT15 (colorectal line). FG88.2, FG88.7 and 505/4 mAbs did not stain AGS glycolipids suggesting the cell line does not express the glycoepitope recognised by these 3 mAbs. In contrast, CA19-9 did recognise a glycolipid in AGS cells. The glycolipid stained by FG88.2 mAb in Colo205 cells showed the same migration exhibited by CA19-9 (R_f = 0.21 and 0.14). In addition, FG88.2 mAb stained another glycolipid with less polarity (R_f = 0.46). In contrast, one of the glycolipids in Colo205 stained by FG88.7 mAb showed the same migration exhibited by 505/4 (R_f = 0.19). FG88.7 mAb also stained an extra glycolipid with less polarity (R_f = 0.29).

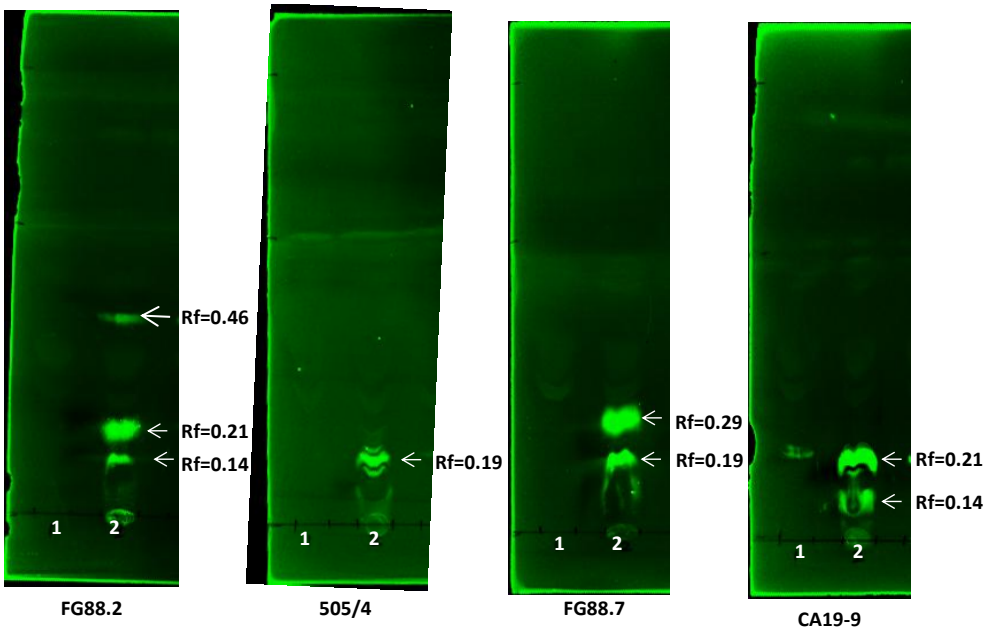


Figure 3.7: Binding of FG88.2 and FG88.7 mAbs to lipid antigens was assessed by thin layer chromatography analysis. Thin layer chromatography analysis of 1) AGS and 2) Colo205 total lipid extracts (2x10^{6} cells equiv.) using FG88.2, 505/4, FG88.7 and CA19-9 mAbs, all at the concentration of 3.6 x 10^{-8} M. (Rf value= migration distance of spot/ migration distance of solvent).

3.2.2.3: FG88 mAbs recognise Le^{c}-and Le^{a}-containing glycans with high functional affinity

To identify the antigen recognised by FG88.2 and FG88.7 mAbs, they were screened against HSA-coupled Le^{a}, Le^{b}, Le^{x} and Le^{y} antigens by ELISA at concentration of 3.6 x 10^{-8} M. 505/4 (anti-sialyl-di-Le^{a}/Le^{a}; 1.95 OD units), FG27.10 (anti-Le^{y}; 1.77 OD units), 225-Le (anti-Le^{b}; 0.64 OD units) and P12 (anti-Le^{x}; 0.89 OD units) mAbs were included as positive controls at concentration of 3.6 x 10^{-8} M. Secondary antibody (anti-mouse IgG-HRPO) alone did not show any reactivity with any glycans. ELISA results showed both FG88.2 and FG88.7 mAbs did not recognise Le^{b} and Le^{y} but strongly recognised

Le^a (FG88.2: 1.74; FG88.7: 1.87 OD units) and weakly recognised Le^x (FG88.2: 0.43; FG88.7: 0.25 OD units; Figure 3.8).

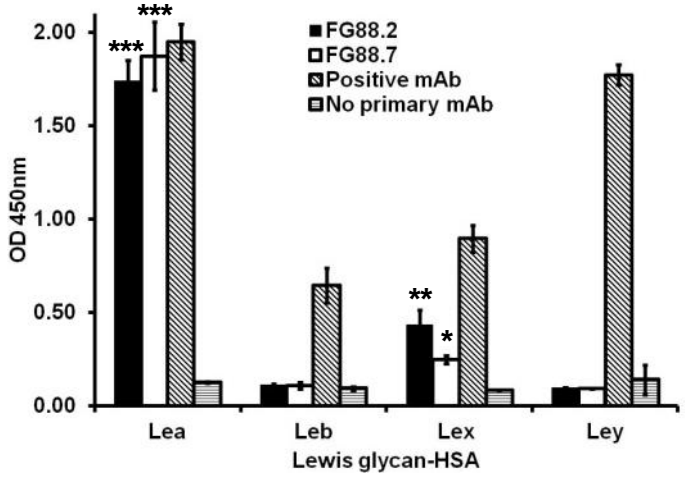


Figure 3.8: Reactivity of FG88.2 and FG88.7 mAbs with HSA coupled Lewis antigens (a, b, x and y). Binding of FG88.2 and FG88.7 mAbs (3.6 x 10^{-8} M) to HSA coupled Lewis antigens were assessed by ELISA. 505/4, P12, FG27.10 and 225-Le mAbs were included as positive controls for Lewis a, x, y and b respectively. Anti-mouse IgG HRPO was used as negative control. Antibody activity was measured by absorbance at 405nm. Error bars represent the mean ± STD of quadruplicate wells (*, P< 0.05; **, P<0.01 and *** P<0.001 versus the control; ANOVA followed by the Bonferroni multiple comparisons test, GraphPad Prism 6).

To clarify their fine specificities, FG88.2 and FG88.7 mAbs were screened against the glycan array from the Consortium for Functional Glycomics (CFG). This glycan array has 610 glycan targets with amino linkers printed onto N-hydroxysuccinimide (NHS)-activated glass microscope slides, forming covalent amide linkages. Binding of FG88.2 and FG88.7 mAbs to the glycan array confirmed the HSA-Lewis ELISA result, with FG88.2 binding most strongly to Le^{a}Le^x, followed by Le^{c}Le^x, Le^a containing glycan, Le^a and Di-Le^a. FG88.7 bound most strongly to Di-Le^a, followed by Le^{c}Le^x, Le^a containing glycans and Le^{a}Le^x (Figure 3.9 and Table 3.2). In addition, the mAbs bound simple Le^a on the array but not Le^c or Le^x. This was corroborated by competition experiments in which pre-incubation of both mAbs with the Le^a-HSA conjugate, fully inhibited Colo205 binding (Figure 3.10), with Le^x-HSA being less effective. Next, Le^a-HSA binding kinetics of FG88 mAbs were examined by using SPR (Biacore X). Fitting of the binding curves revealed strong apparent functional affinity (K_d ~ 10^{-10} M) with fast association (~ 10^5 1/Ms) and slow dissociation (~10^{-5} 1/s) rates for both mAbs (Table 3.3). This was in line with EC_{50} values from Le^a-HSA ELISA (Figure 3.11 and Table 3.3). Cellular functional affinity was lower (K_d ~ 10^{-9}; Figure 3.12 and Table 3.3), reflecting the complex mAb binding at the

cell surface with mixed glycolipid/glycoprotein recognition and the possibility of secondary events (internalisation or cell killing).

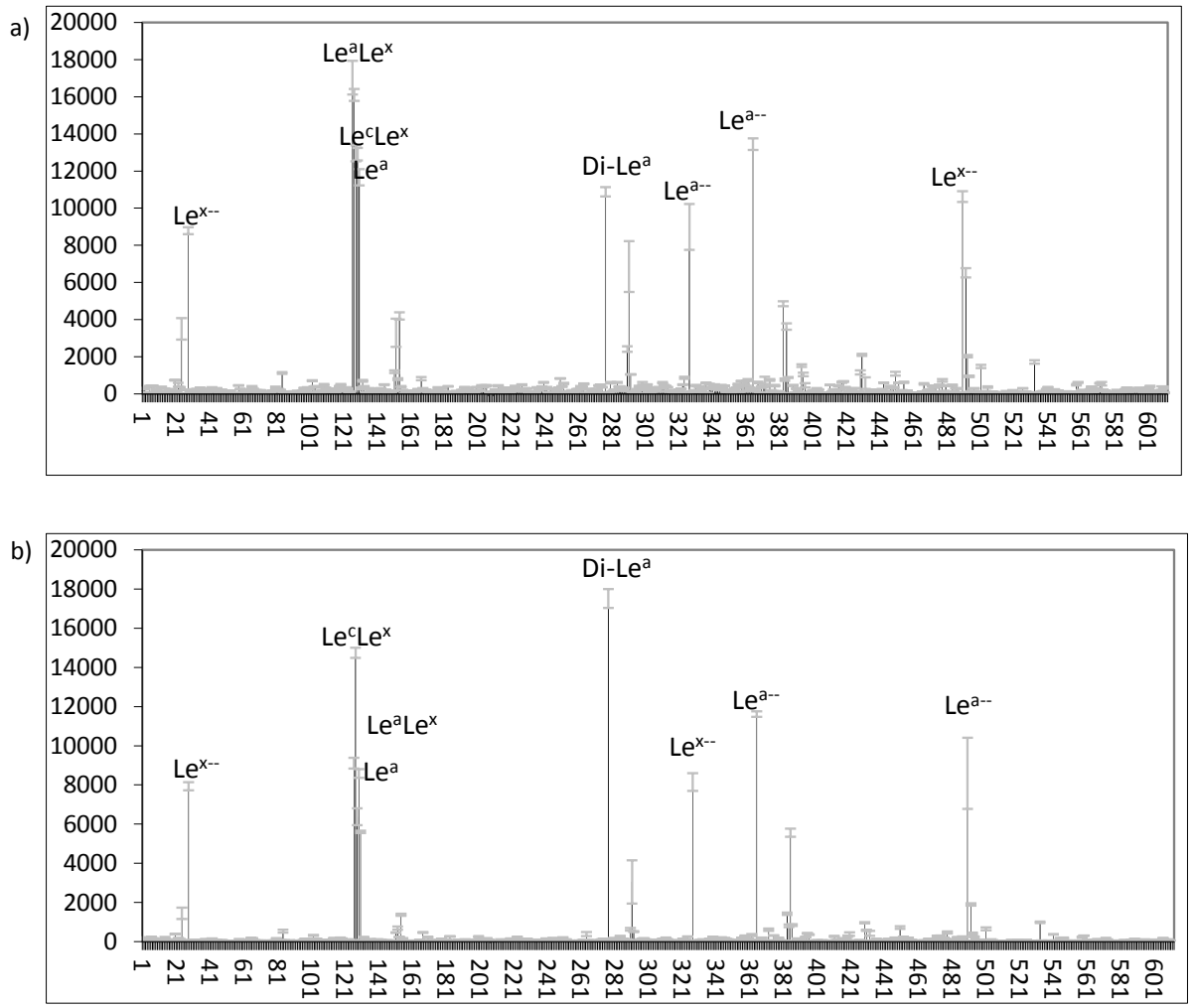
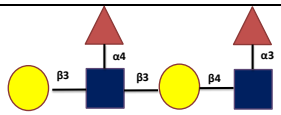
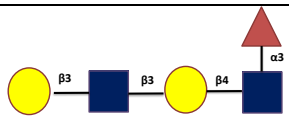
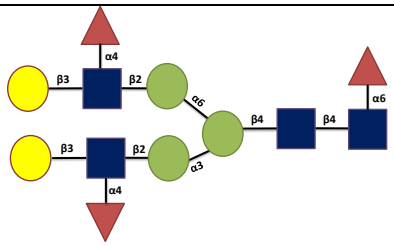
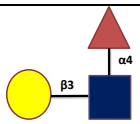
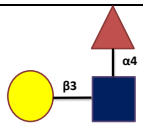
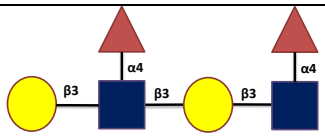
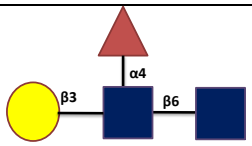
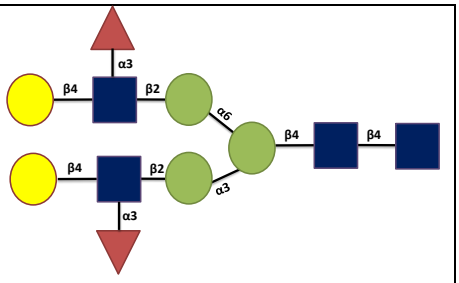


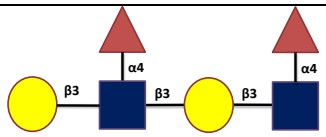
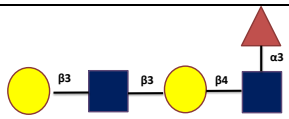
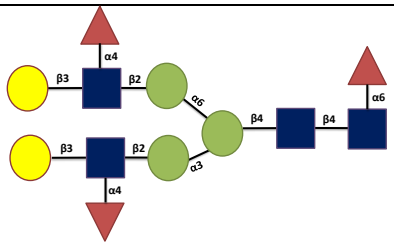
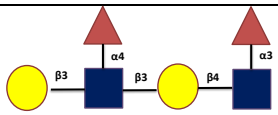
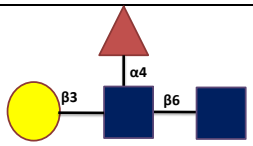
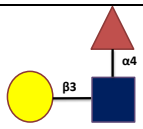
Figure 3.9: Binding of FG88.2 and FG88.7 mAbs to The Consortium for Functional Glycomics (CFG) glycan array. FG88.2 and FG88.7 mAbs were screened against the CFG glycan array which is composed of 610 mammalian glycan targets by ELISA. The fine specificity between a) FG88.2 mAb and b) FG88.7 mAb were compared; where a= Lewis a; c= Lewis c; x= Lewis x; Le^a--= Le^a containing glycan and Le^x--= Le^x containing glycan

a)

Glycan number	Glycan	Name	% of best binder	Structure
126	Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	Le^aLe^x	100	
127	Galb1-3GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	Le^cLe^x	94	
365	Fuca1-4(Galb1-3)GlcNAcb1-2Mana1-6(Fuca1-4(Galb1-3)GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp22	Le^a containing glycan	79	
128	Galb1-3(Fuca1-4)GlcNAc-Sp0	Le^a	76	
129	Galb1-3(Fuca1-4)GlcNAc-Sp8	Le^a	76	
277	Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-3(Fuca1-4)GlcNAcb-Sp0	Di-Le^a	64	
490	Galb1-3(Fuca1-4)GlcNAcb1-6GalNAca-Sp14	Le^a containing glycan	62	

327	Galb1-4(Fuca1-3)GlcNAcb1-2Mana1-6(Galb1-4(Fuca1-3)GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp20	Le^x containing glycan	53	

b)

Glycan number	Glycan	Name	% of best binder	Structure
277	Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-3(Fuca1-4)GlcNAcb-Sp0	Di-Le^{a}	100	
127	Galb1-3GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	Le^{c}Le^{x}	84	
365	Fuca1-4(Galb1-3)GlcNAcb1-2Mana1-6(Fuca1-4(Galb1-3)GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp22	Le^{a} containing glycan	66	
126	Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	Le^{a}Le^{x}	52	
490	Galb1-3(Fuca1-4)GlcNAcb1-6GalNAca-Sp14	Le^{a} containing glycan	49	
129	Galb1-3(Fuca1-4)GlcNAc-Sp8	Le^{a}	49	

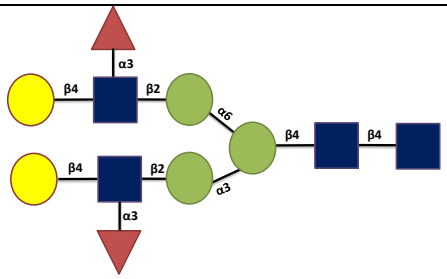
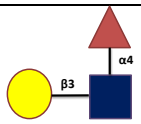
327	Galb1-4(Fuca1-3)GlcNAcb1-2Mana1-6(Galb1-4(Fuca1-3)GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp20	Le^x containing glycan	47	
128	Galb1-3(Fuca1-4)GlcNAc-Sp0	Le^a	36	

Table 3.2: Details of glycan binding by a) FG88.2 and b) FG88.7 to the CFG glycan array.

represents glucosylamine, represents galactose, represents fucose, represents mannose. Sp denotes the length of spacer between glycan and slide.

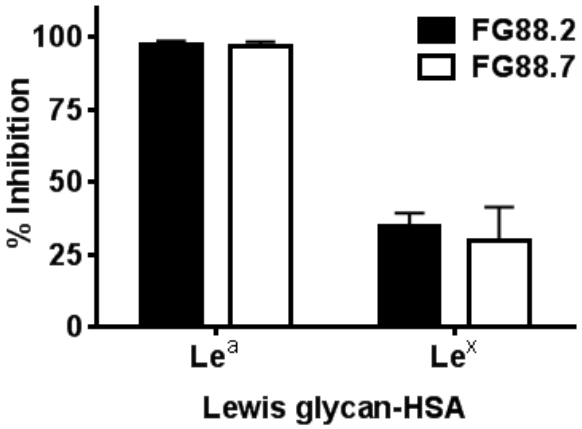


Figure 3.10: Lewis glycan competition analysis. HCT15 cells were incubated with 2.2 x 10^{-8} M FG88 mAbs, preincubated with a 40-fold molar excess of Le^a-HSA or Le^x-HSA, or PBS (control). Cell binding was analysed using indirect immunofluorescence and flow cytometric analysis and the results (mean ± STD from 3 independent experiments) are presented as percentage inhibition of the cell binding of the control.

	SPR Real-time Le^a-HSA binding			FACS cell surface binding	ELISA Lea-HSA binding
mAb	Association rate K_{on} (1/Ms)	Dissociation rate K_{off} (1/s)	Dissociation constant K_d (M)	Dissociation constant K_d (M)	Half maximal effective concentration EC_{50} (M)
FG88.2	1.9×10^5	1.2×10^{-4}	0.6×10^{-9}	7.4×10^{-9}	1.1×10^{-9}
FG88.7	1.7×10^5	5.8×10^{-5}	0.3×10^{-9}	5.7×10^{-9}	0.7×10^{-9}

Table 3.3: Determination of kinetic and equilibrium Le^a-binding parameters SPR, FACS and ELISA. Binding curves from which the Kd and EC_{50} values are deduced are shown in Figures 3.13 and 3.14, respectively. Representative results from at least two independent experiments are listed.

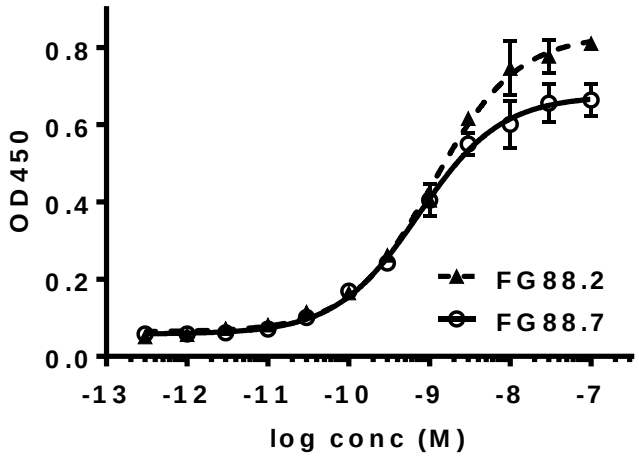


***Figure 3.11: Le^a-HSA ELISA*.** Binding of a range of concentrations of the FG88 mAbs to Le^a-HSA was assessed by ELISA. Representative binding curves from two independent experiments are shown. EC_{50} values were obtained via non-linear regression on log-transformed data (GraphPad Prism 6).

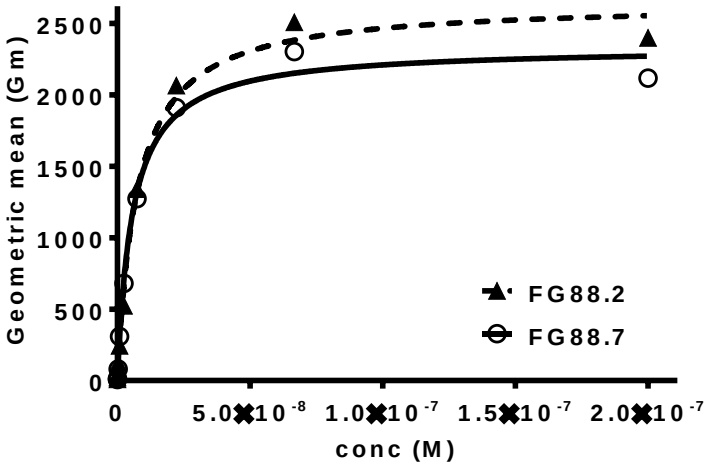


Figure 3.12: HCT15 cell surface binding. HCT15 cells were incubated with a concentration range of FG88 or isotype control mAbs and cell binding analysed using indirect immunofluorescence and flow cytometric analysis. Fitting of the background - subtracted data to a one site specific binding model (GraphPad Prism 6) generated the K_d values. Representative binding curves from three independent experiments are shown.

3.2.2.4: FG88.2 mAb exhibits strong tumour reactivity, with FG88.2 staining associated with reduced colorectal and breast cancer patient survival; and with colorectal staining being an independent prognostic marker

The two FG88 mAbs differ by only three residues, two in the CDRs and one in the FR region. Therefore, FG88.2 mAb was chosen to stain colorectal, breast, ovarian, gastric, NSCLC and pancreatic tumour TMAs,in order to assess the possible targets for FG88.2 mAb (Table 3.5). FG88.2 mAb stained 71% (147 of 208) of colorectal, 27% (243 of 902) of breast, 31% (66 of 217) of ovarian, 54% (52 of 96) of gastric, 23% (62 of 274) of non-small cell lung cancer (NSCLC) and 81% (155 of 192) of pancreatic tumours. Examples of FG88.2 staining level of tumour tissues are shown (Figure 3.13). No significant association between FG88.2 glycoepitope expression and clinicopathologic variables was observed in ovarian, gastric, NSCLC and pancreatic TMAs.

Table 3.5: FG88 glycoepitope expression on human tumour types						
Tumour type	**Total**	**Positive (%)**	**3+**	**2+**	**1+**	**0**
Colorectal	208	71% 147/208	24	74	49	61
Gastric	96	54% 52/96	13	24	15	44
Pancreatic	248	81% 196/248	24	108	64	52
NSCLC	274	23% 62/274	10	15	37	212
Ovarian	217	31% 66/217	19	19	28	150
Breast	902	27% 110/902	7	28	75	792

Human tumor tissues were stained with FG88.2 (2.7×10^{-9} M). The staining intensity was scored according to: negative (0), weak (1), moderate (2) and strong (3). (*NSCLC: non-small cell lung cancer*)

In the colorectal cohort (S. A. Watson et al., 1993), FG88.2 glycoepitope expression was significantly associated with fucosyltransferase 3 (FUT3) expression (χ^2 = 26.712; *P* = 0.031). The Kaplan-Meier analysis of disease-free survival of colorectal cancer patients revealed a significantly lower mean survival time in the high FG88.2 glycoepitope expressing group [mean survival, 36 months (high; n = 27) versus 79 months (low; n = 184); *P* = 0.01; Figure 3.14]. On multivariate analysis using Cox regression, high FG88.2 glycoepitope expression in colorectal cancer was a marker of poor prognosis which was independent of stage and vascular invasion (*P*< 0.001).

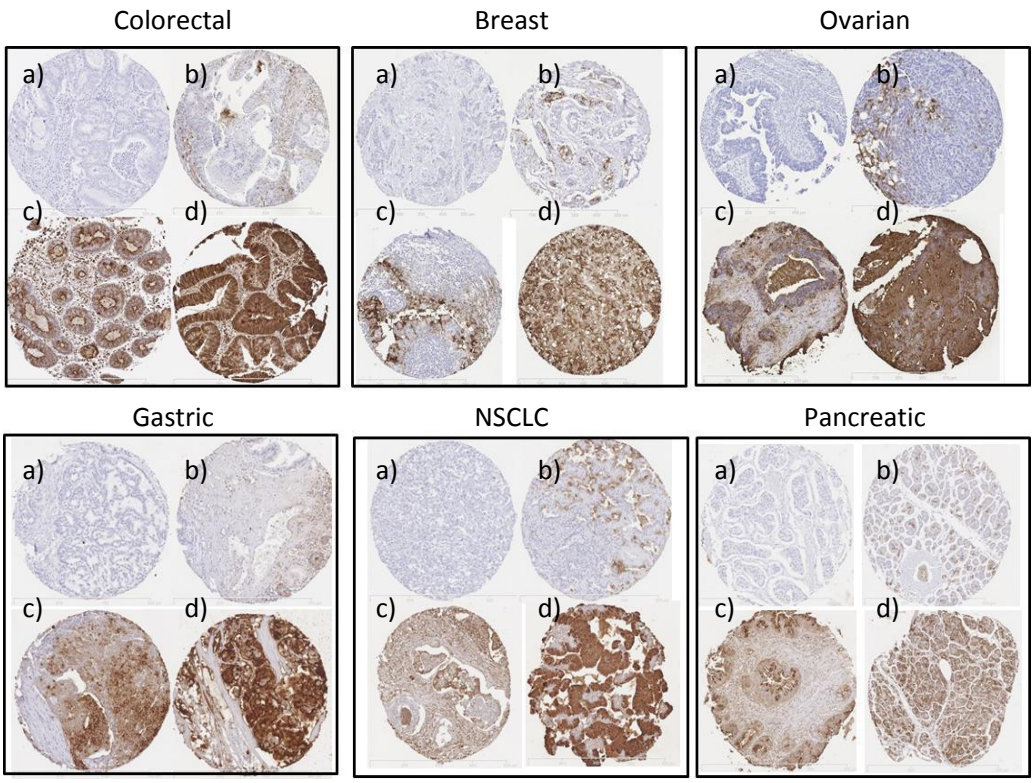


Figure 3.13: FG88.2 tumour tissue staining. Colorectal, breast, ovarian, gastric, NSCLC and pancreatic TMAs were stained with FG88.2 mAb at 2.7 x 10^{-9} M. Representative images of a) negative, b) weak, c) moderate and d) strong FG88.2 staining are shown (*magnification x20*).

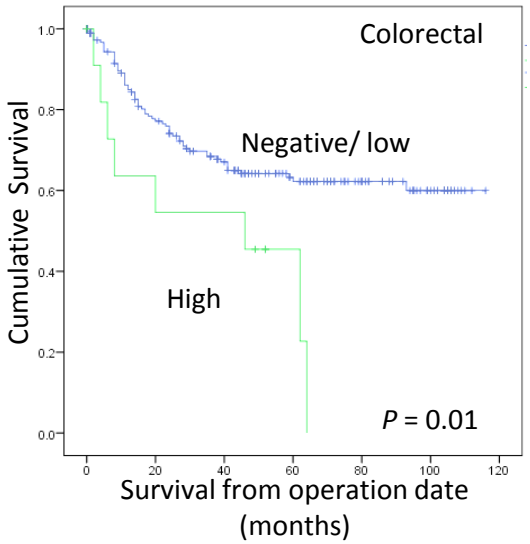


Figure 3.14: Survival analysis. Kaplan-Meier analysis of progression-free survival in colorectal cancer TMA cohort showing the impact of FG88.2 glycoepitope expression. Significance was determined using the log-rank test.

In the breast cohort, FG88.2 glycoepitope expression was directly correlated with larger primary tumour size (≥2.0cm; P = 0.001), high tumour grade (*P*< 0.001), high tubule formation (*P* = 0.034),

high pleomorphism (*P*< 0.002), high mitosis (*P*< 0.001), TNBC (*P*< 0.001), BL-TNBC (*P*< 0.001) and inversely correlated with ER (*P*< 0.001), PgR (*P*< 0.001) and HER2 (*P*< 0.001). FG88.2 glycoepitope expression was not associated with age, vascular invasion, stage, c-erb-B3 expression, c-erb-B4 expression and mucin I expression. The Kaplan-Meier analysis of disease-free survival of breast cancer patients revealed the mean survival time is significantly lower in the high FG88.2 glycoepitope expressing group (*P* = 0.008, log rank test; n=7.029) (Figure 3.15). Mean survival for high FG88.2 glycoepitope expressing group was 190 months and 217 months for negative or the low FG88.2 glycoepitope expressing group. A Cox regression model was constructed to assess whether FG88.2 glycoepitope expression was an independent prognostic factor. High tumour stage (*P*< 0.001, HR= 1.553, 95% CI = 1.315-1.834), grade (*P*< 0.001, HR= 1.338, 95% CI = 1.123-1.594) and size (p<0.001, HR= 0.665, 95% CI = 1.174-1.925) were significantly associated with poor survival. Patients whose tumours express high levels of FG88.2 glycoepitope showed a trend towards reduced progression-free survival which did not reach statistical significance in this cohort (*P* = 0.07, HR= 1.34, 95% CI = 0.976-1.84).

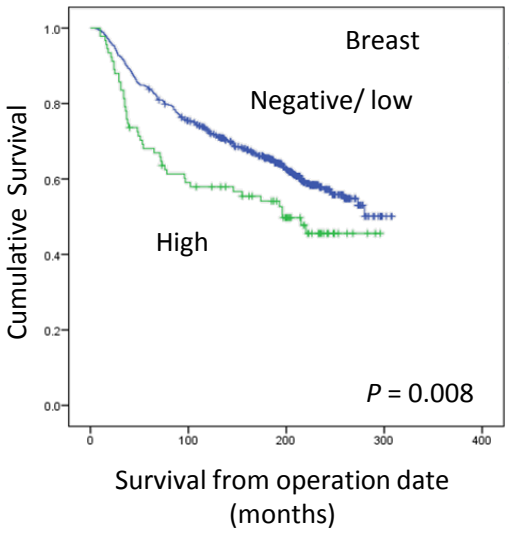


Figure 3.15: Survival analysis. Kaplan-Meier analysis of progression-free survival in breast cancer TMA cohort showing the impact of FG88.2 glycoepitope expression. Significance was determined using the log-rank test.

In patients with ER (+) breast tumours (677 cases), high FG88.2 glycoepitope expression was significantly associated with reduced patient survival (*P* = 0.003, X^2 = 8.676) (Figure 3.16). Mean disease-free survival for high FG88.2 glycoepitope expression patients was 195 months and 207 months for negative or low FG88.2 glycoepitope expression.

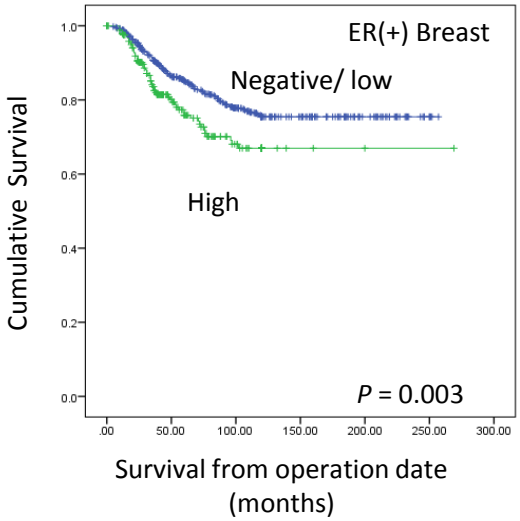


Figure 3.16: Survival analysis. Kaplan-Meier analysis of progression-free survival in ER (+) breast cancer TMA cohort showing the impact of FG88.2 glycoepitope expression. Significance was determined using the log-rank test.

3.2.3: FG88 mAbs exhibit limited normal tissue cross reactivity and do not bind PBMCs, granulocytes and erythrocytes

To assess the possible toxicity of FG88.2 and FG88.7 mAbs, human and cynomolgus monkey normal tissue TMAs were stained. For human normal tissue TMA, FG88.2 mAb did not stain placenta, rectum, skin, adipose, heart, skeletal, bladder, spleen, brain, stomach, breast, kidney, testis, cerebellum, cervix, lung, ovary, diaphragm, uterus, duodenum and thyroid. Staining was seen against oesophagus (weak squamous epithelium staining), gall bladder (weak to moderate columnar epithelium staining), Ileum (moderate columnar mucosa staining), jejunum (weak columnar mucosa staining), liver (moderate bile duct staining), thymus (weak staining), colon (moderate glandular epithelium staining), tonsil (weak to moderate squamous epithelium staining) and pancreas (weak staining). FG88.7 mAb showed the same staining pattern as FG88.2 mAb except that it also stained normal rectum (weak glandular epithelium staining) (Figure 3.17a, b and Table 3.4). Similar but not identical staining of the monkey normal tissue TMA, was observed, with binding to small intestine, skin, colon, stomach, ovary, liver and thymus for both FG88.2 and FG88.7 mAbs (Figure 3.18).

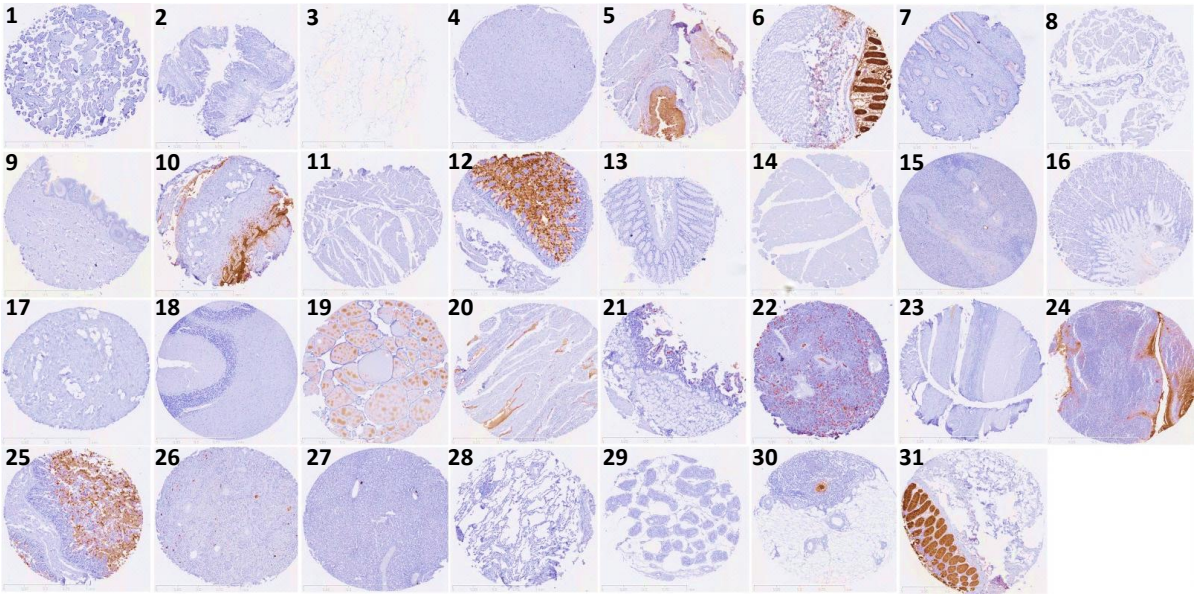


Figure 3.17a: Normal human tissue staining. AMSBIO normal human tissue microarray was stained by ABC-immunohistochemistry with murine FG88.2 (2 x 10^{-9} M) counterstained with hematoxylin. 1) placenta; 2) bladder; 3) adipose; 4) brain; 5) esophagus; 6) colon; 7) cervix; 8) diaphragm; 9) skin; 10) gall bladder; 11) heart; 12) ileum; 13) rectum; 14) skeletal muscle; 15) spleen; 16) stomach; 17) breast; 18) cerebellum; 19) thyroid; 20) uterus; 21) duodenum; 22) pancreas; 23) ovary; 24) tonsil; 25) jejunum; 26) kidney; 27) liver; 28) lung; 29) testis; 30) thymus and 31) small intestine (*magnification x20*).

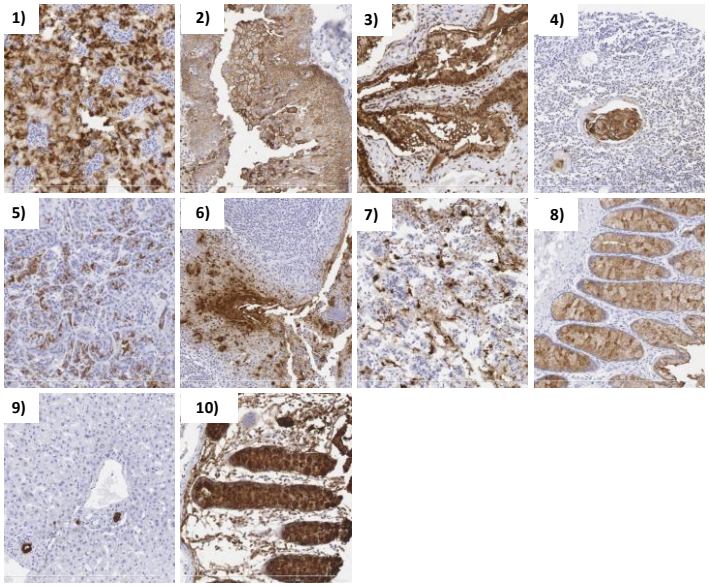


Figure 3.17b: Subcellular location of FG88.2 glycoepitope antigens on representative human normal tissues. AMSBIO normal human tissue microarray was stained by ABC-immunohistochemistry with murine FG88.2 (2 x 10^{-9} M) and counterstained with haematoxylin. 1) Ileum, exhibiting moderate columnar mucosa staining; 2) Oesophagus, exhibiting weak squamous

epithelium staining; 3) gall bladder, exhibiting weak to moderate columnar epithelium staining; 4) thymus, exhibiting weak staining; 5) pancreas, exhibiting weak staining; 6) tonsil exhibiting weak to moderate squamous epithelium staining; 7) jejunum, exhibiting weak columnar mucosa staining; 8) rectum, exhibiting weak glandular epithelium staining ; 9) liver, exhibiting moderate bile duct staining; 10) colon exhibiting moderate glandular epithelium staining (*magnification ×20*).

Table 3.4: Normal human tissue staining by FG88 mAbs

Tissue	**FG88.2**	**FG88.7**	**Tissue**	**FG88.2**	**FG88.7**
Placenta	0	0	Testis	0	0
Oesophagus	1	0/1	Cerebellum	0	0
Rectum	0	1	Liver	0/1	0/1
Gall bladder	1/2	1	Thymus	0/1	0/1
Skin	0	0	Cervix	0	0
Adipose	0	0	Lung	0	0
Heart	0	0	Small intestine	0/2	0/2
Skeletal muscle	0	0	Colon	0/2	0/2
Bladder	0	0	Ovary	0	0
Ileum	0/2	0/1	Tonsil	1	1/2
Spleen	0	0	Diaphragm	0	0
Brain	0	0	Pancreas	0/1	0/1
Jejunum	1/2	1	Uterus	0	0
Stomach	0	0	Duodenum	0	0
Breast	0	0	Thyroid	0	0
Kidney	0	0			

Normal human tissue TMA (AMSBIO) was stained with FG88 mAbs (2 x 10^{-9} M). The staining intensity was scored according to: negative (0), weak (1), moderate (2) and strong (3).

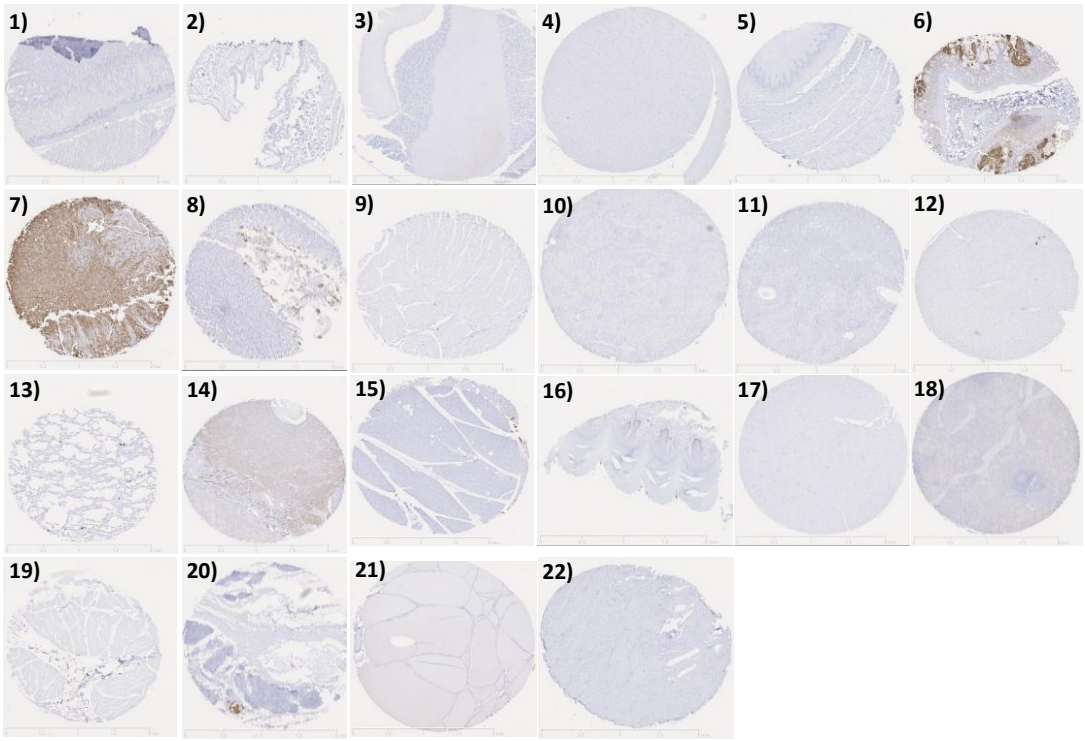


Figure 3.18: FG88.2 binding to normal cynomolgus monkey TMA. Normal cynomolgus monkey TMA (AMSBIO) was stained with FG88.2 (2 x 10^{-9} M) and counterstained with hematoxylin. 1) adrenal gland; 2) bladder; 3) cerebellum; 4) cerebral cortex; 5) esophagus; 6) stomach; 7) small intestine; 8) colon; 9) heart; 10) kidney cortex; 11) kidney medulla; 12) liver; 13) lung; 14) ovary; 15) pancreas; 16) skin; 17) spinal cord; 18) spleen; 19) striated muscle; 20) thymus, 21) thyroid and 22) uterus (*magnification x 20*).

As previous studies had shown that Le^x was expressed on neutrophils, it was of interest to check whether FG88.2 and FG88.7 mAbs bound to any peripheral blood mononuclear cells (PBMCs) and/or granulocytes. Thus, binding of FG88 mAbs to 3 healthy donor whole bloods was analysed by FACS (Figure 3.19). 505/4 (anti-sialyl-di-$Le^{a/}Le^{a}$), CA19-9 (anti-SLe^a) and 7-Le (anti-Le^a) were included in the same panel for comparison. W6/32 (anti-HLA-A, B, C), CEACAM6 (anti-CEACAM6; granulocytes) and OKT3 (anti-CD3: T cells) mAbs were used as positive controls. IgG isotype control and medium alone were included as negative controls. As shown in Figure 3.19, FG88.2, FG88.7, 505/4, 7-Le and CA19-9 mAbs did not stain either PBMCs or granulocytes from 3 healthy donors, whereas anti-CEACAM6 bound strongly to granulocytes and anti-CD3 to lymphocytes from all three donors. In addition, Le^a and Le^b antigens found in tissue secretions can be adsorbed onto erythrocytes. By using FG88.2 mAb, the secretor status of 9 healthy human donors was determined by saliva sandwich ELISA (Figure 3.20). Donor 3, 5 and 6 were secretors and may therefore have Le^a and Le^b antigens

absorbed to their erythrocytes. However, neither FG88 mAb bound to erythrocytes from either secretor or non-secretor donors (Figure 3.21).

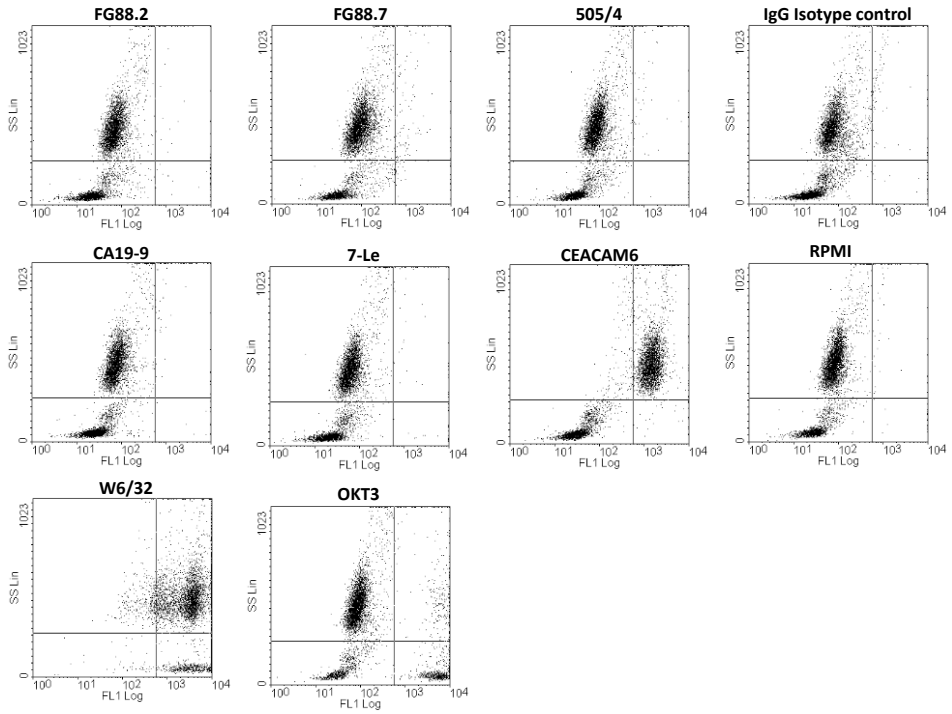


Figure 3.19: Binding of FG88.2 and FG88.7 mAbs to whole blood from healthy donors. Binding of healthy donor whole bloods with FG88.2, FG88.7, 505/4, CA19-9 and 7-Le mAbs was assessed by indirect immunofluorescent staining and flow cytometric analysis. Binding of these mAbs to whole bloods was compared to the positive control mAbs, W6/32 (anti-HLA-A, B, C); CEACAM6 (anti-CEACAM6); and OKT3 (anti-CD3). Mouse immunoglobulin (IgG) and medium alone were used as negative controls. All mAbs were used at 7 x 10^{-8} M. The result shown is representative of 3 different healthy donor whole bloods.

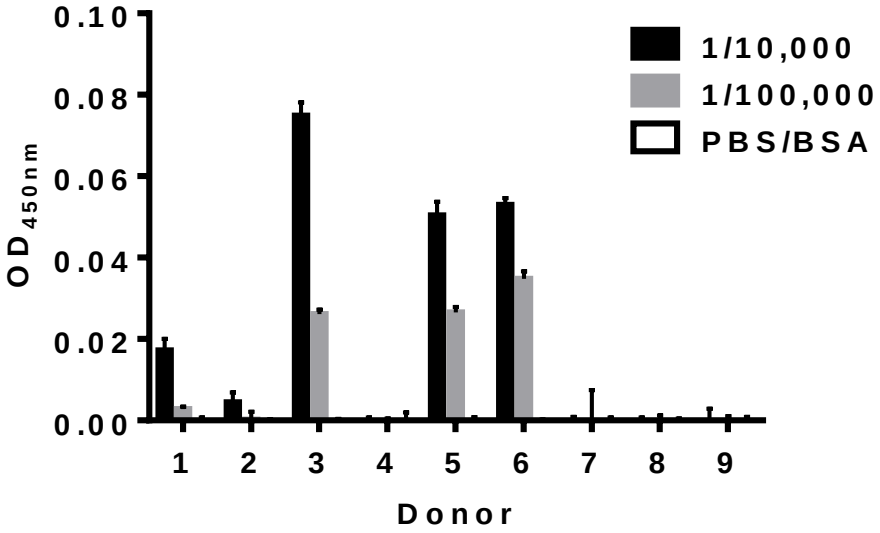


Figure 3.20: Sandwich ELISA using FG88.2 for the detection of secreted Le^a in saliva. Saliva from nine healthy donors was collected, heat-inactivated and analysed for the presence of Le^a by sandwich ELISA. Three out of nine donors tested positive.

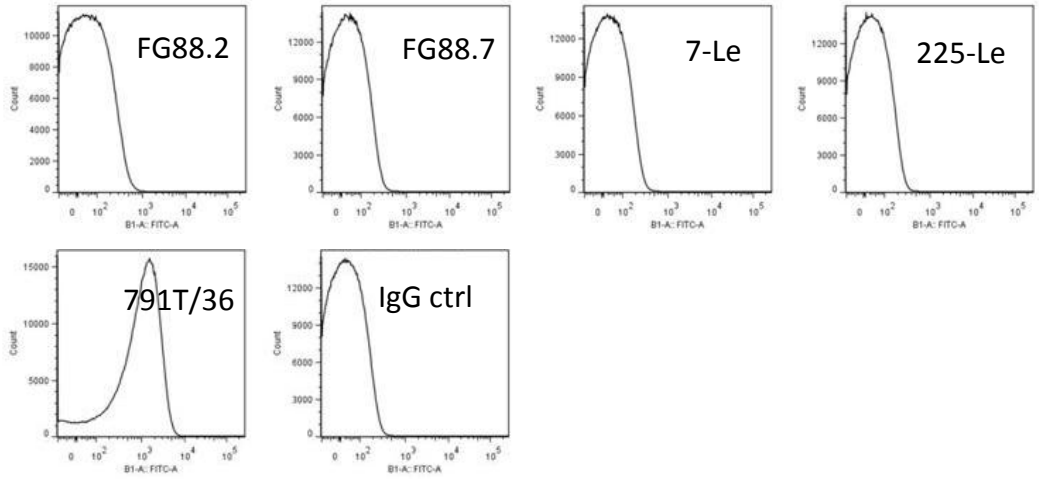


Figure 3.21: Evaluation of FG88 mAbs binding to erythrocytes from a representative Le^a-positive donor by flow cytometry. Binding of healthy Le^a-positive donor red blood cells with FG88.2, FG88.7, 7-Le, 225-Le and 791T/36 mAbs was assessed by indirect immunofluorescent staining and flow cytometric analysis. Absence of erythrocyte binding by FG88.2 and FG88.7 mAbs was compared to the control mAbs; 7-Le (anti-Le^a) and 225-Le (anti-Le^b). MAb 791T/36 (anti-CD55) was included as a positive control and an IgG isotype (IgG ctrl) was used as the negative.

3.2.4: FG88 mAbs as therapy for colorectal cancer

3.2.4.1: FG88 mAbs mediate direct tumour cytotoxicity via a unique caspase independent mechanism

As it is difficult to use primary tumours for cytotoxic experiments due to their high rate of spontaneous apoptosis, cancer cell lines were used as models for FG88 mAbs binding and killing.

Binding of FG88.2 mAb to a panel of tumour cell lines (Figure 3.22) was studied by flow cytometry. FG88.2 bound strongly to HCT15 and Colo205 (Gm ≥ 1,000 at saturating mAb concentration). Moderately binding cells (Gm ≥ 100) included HT29 and OVCAR3; low binding cells (Gm < 100) included AGS and DMS79.

To ascertain whether cancer cell binding by the FG88 mAbs affected cell viability, FG88 mAbs were tested for direct induction of PI uptake in HCT15 cells at 37°C, which reflects compromised membrane integrity and loss of cell viability (Figure 3.23). For comparison, 505/4 mAb (that is known to induce cell death) was also included. Medium alone was included as negative control. These 3 murine mAbs induced cell death at antibody concentrations of 7.3 x 10^{-8} M and 1.4 x 10^{-7} M. 505/4 induced 72.8% (1.4 x 10^{-7} M) and 87.6% (7.3 x 10^{-8} M) PI uptake, FG88.2 induced 75.9% (1.4 x 10^{-7} M) and 76.3% (7.3 x 10^{-8} M) PI uptake and FG88.7 induced 82.3% (1.4 x 10^{-7} M) and 82% (7.3 x 10^{-8} M) PI uptake. Cells incubated with medium alone showed 21.3% and 21.1% PI uptake.

a)

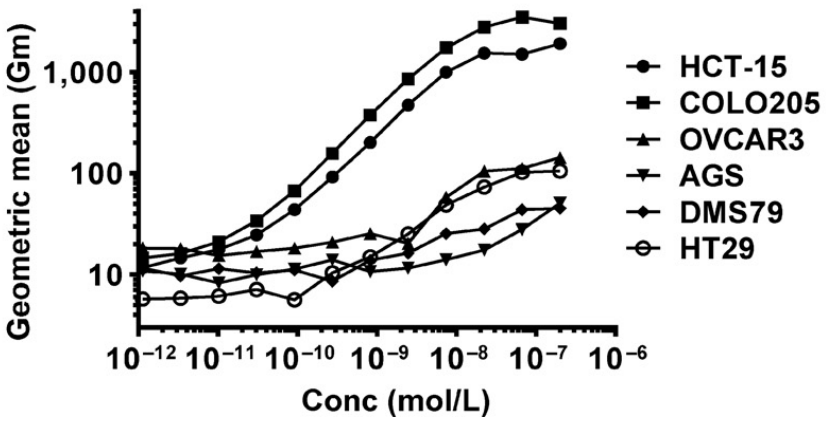


Figure 3.22: FG88.2 tumour cell binding by flow cytometry. a) Binding of FG88.2 mAb was assessed by indirect immunofluorescent staining and flow cytometric analysis. FG88.2 binding to a panel of tumour cell lines in the concentration range 1.1 x 10^{-12} – 2.2 x 10^{-7} M, is presented as geometric means (Gm).

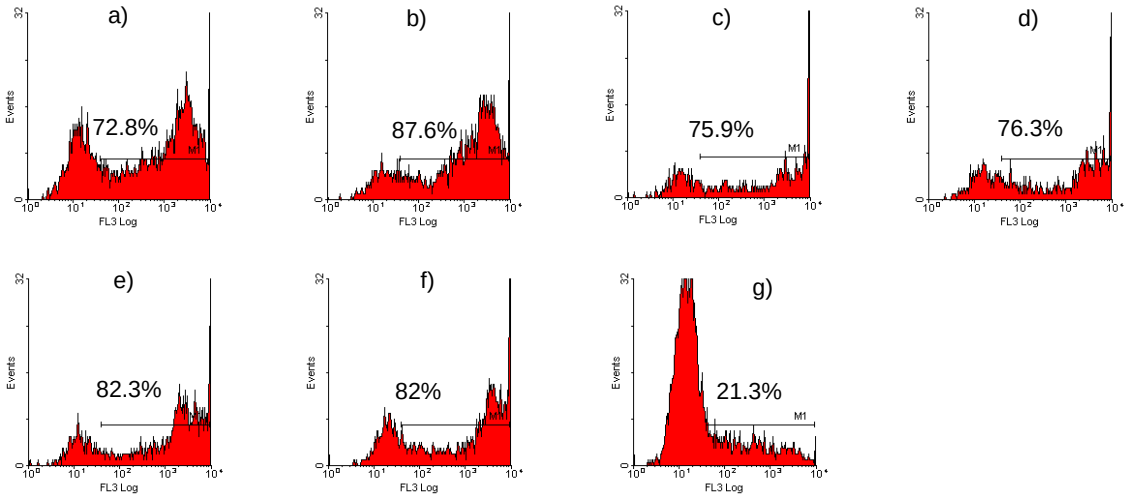


Figure 3.23: FG88.2 and FG88.7 mAbs induced direct cell death of HCT15 cells at 37^{0}C. PI uptake following mAb exposure was assessed by flow cytometric analysis. HCT15 cells were incubated with 7.3 x 10^{-8} M and 1.4 x 10^{-7} M of FG88.2 and FG88.7 mAbs at 37^{0}C. 505/4 mAb and medium alone were included as positive and negative control respectively. a) 505/4 mAb (1.4 x 10^{-7} M), b) 505/4 mAb (7.3 x 10^{-8} M), c) FG88.2 mAb (1.4 x 10^{-7} M), d) FG88.2 mAb (7.3 x 10^{-8} M), e) FG88.7 mAb (1.4 x 10^{-7} M), f) FG88.7 mAb (7.3 x 10^{-8} M), g) medium alone.

To assess direct killing on cells with varying expression of FG88 glycoepitopes, PI uptake assay was carried out using FG88.2 mAb with Colo205, HCT15, HT29, OVCAR3, DMS79 and AGS cells (Figure 3.24). FG88.2 induced dose-dependent PI uptake, with strong binding cells (such as Colo205 and HCT15), being more susceptible than the moderate to weak FG88-binding cells, suggesting a correlation between killing efficiency and the level of FG88 glycoepitope expression. Cells expressing low to moderate levels of glycoepitope did not display PI uptake.

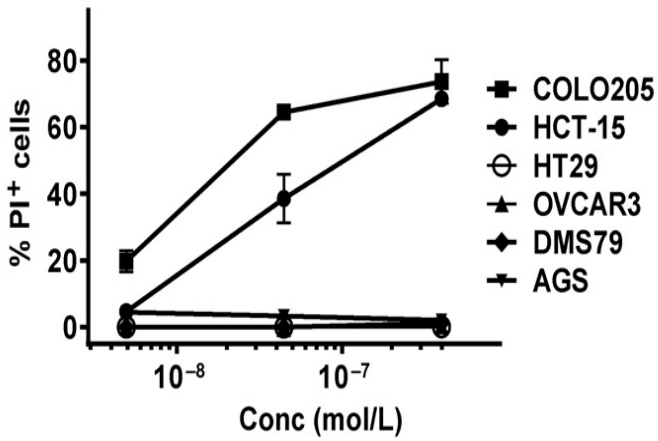


Figure 3.24: Direct cytotoxic activity of FG88.2 mAb. PI uptake following mAb exposure was assessed by flow cytometric analysis. Dose-dependent increase in PI uptake by a range of tumour cell lines as a result of FG88.2 binding (2.4 x 10^{-9} to 2.2 x 10^{-7} M).

To confirm that the PI uptake assay truly reflected cell death in growing cells, the cell viability of HCT15 cells treated with FG88.2 and FG88.7 mAbs was evaluated by light microscopy. FG27.18 (anti-$Le^{y/x}$; 7.3 x 10^{-8} M) mAb and medium alone were used as negative controls. FG88 mAbs-treated HCT15 cells showed evidence of monolayer disruption, cell rounding and clumping and decrease in cell numbers between 24 and 48 hr after the addition of the mAb. This was maintained over the 72 hr incubation period. Cells incubated with FG27.18 and medium alone did not show growth inhibition and achieved 100% confluent over the 72 hr incubation period (Figure 3.25). In addition, proliferation analysis (WST8-based) of FG88.2 mAb-treated HCT15 cells revealed a dose-dependent growth inhibition with an IC_{50} of 1.04 x 10^{-8} M (Figure 3.26).

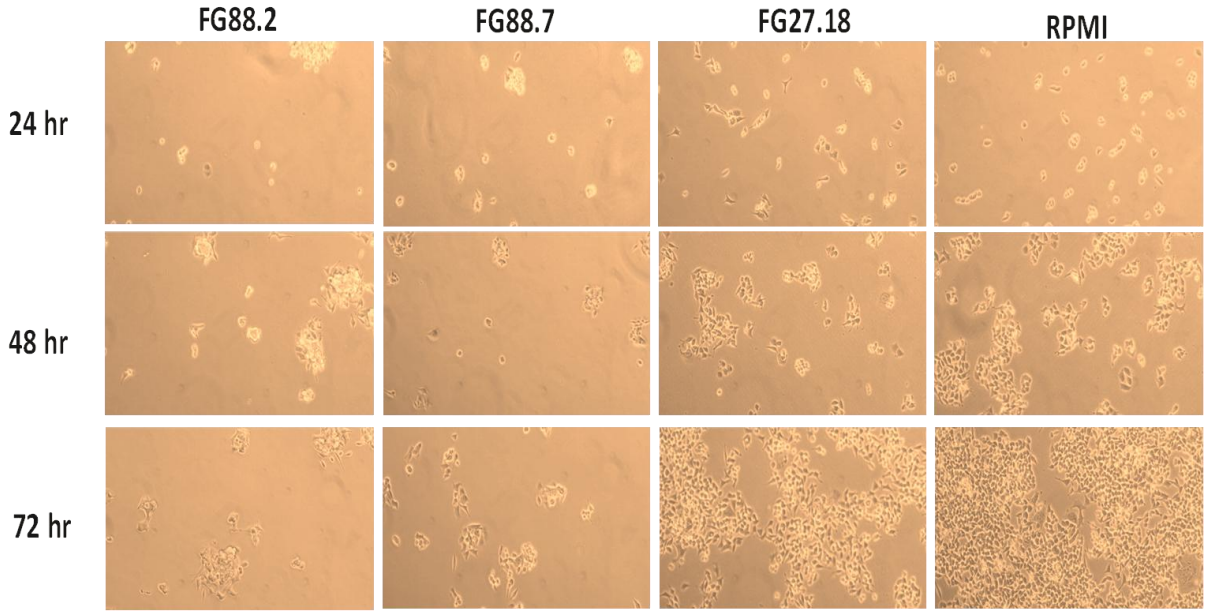


Figure 3.25: Phase-contrast imaging of FG88 mAb-treated HCT15 cells. Light microscopic images (*magnification x10*) showing HCT15 cells after incubation with FG88.2 and FG88.7, both at 2.2 x 10^{-7} M, FG27.18 and medium alone (RPMI) were used as negative controls. Images taken using a Nikon Eclipse TS100.

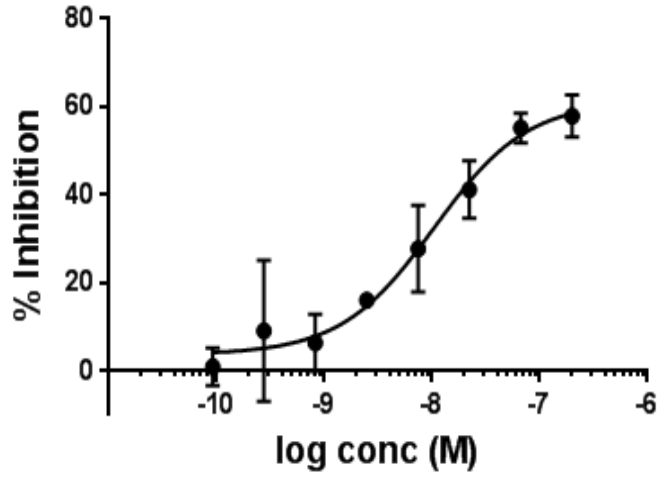


Figure 3.26: Direct cytotoxicity of FG88.2 on HCT15 cells. The anti-proliferative effect of FG88.2 mAb was analysed using the metabolic WST8 assay. HCT15-cells were incubated with FG88.2 mAb for 72 hr, after which the WST-8 reagent was added following the manufacturer's instructions. Results are expressed as percentage inhibition compared to control cells and are representative of two independent experiments.

Classical extrinsic apoptotic cell death involves activation of effector caspases. In order to determine whether the FG88 mAbs inhibited tumour cell growth via apoptosis, metabolically active HCT15 monolayer cells were incubated with FG88 mAbs (3×10^{-10} – 2.4×10^{-8} M) in the presence or absence of 20µM of a cell permeant pan-caspase inhibitor (Z-FMK-VAD). As positive control for apoptotic cell death, Jurkat cells were incubated with anti-Fas mAb (1.8×10^{-8} M) in the presence or absence of Z-FMK-VAD. FG88 mAbs binding to HCT15 cells resulted in a dose dependent decrease in cellular proliferation that was not affected by the presence of Z-FMK-VAD, suggesting no involvement of caspase activity. In contrast, Z-FMK-VAD prevented anti-Fas mAb-mediated apoptosis of Jurkat cells (Figure 3.27).

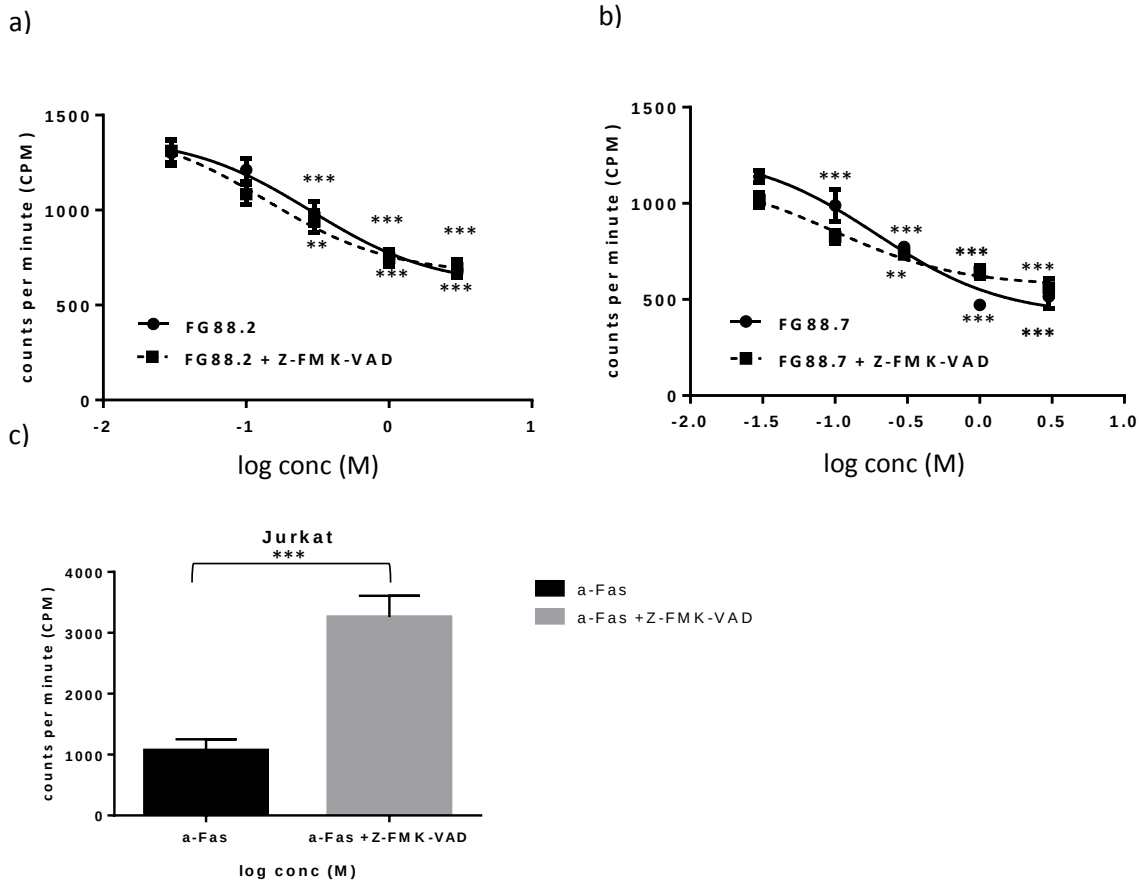
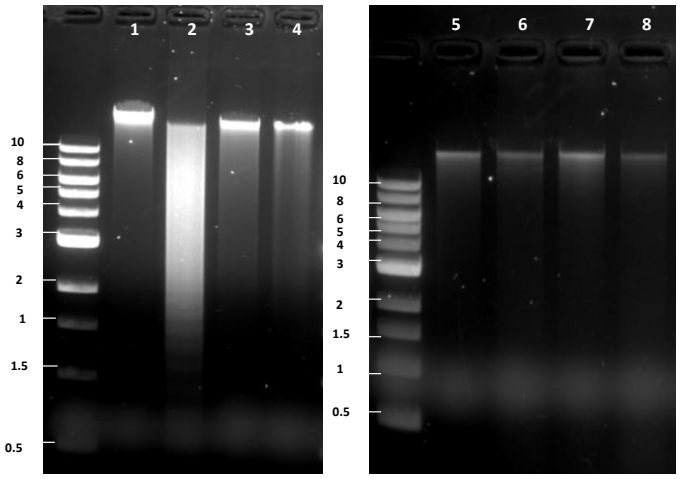


Figure 3.27: FG88 mAbs inhibit HCT15 cell growth in ^{3}H-thymidine assay via caspase independent pathway. a, b) Dose dependent growth inhibition of HCT15 cells by FG88 mAbs (3×10^{-10} – 2.4×10^{-8} M) in the presence or absence of Z-FMK-VAD (20µM). c) Anti-Fas mAb mediated apoptosis of jurkat cells under similar conditions was used as a positive control. Non-linear regression was performed using GraphPad Prism 6 (**, P< 0.01; ***, P< 0.001 versus control).

Another hallmark of apoptotic cell death is endonuclease-induced nuclear DNA fragmentation. Both FG88.2 and FG88.7 did not induce DNA fragmentation in HCT15 cells, in contrast to the anti-Fas mAb that resulted in Z-FMK-VAD-sensitive DNA fragmentation in Jurkat cells (Figure 3.28). The absence of caspase involvement combined with a lack of DNA fragmentation suggests that FG88 mAbs induce a direct cytotoxic effect via a distinct non-apoptotic mechanism.

To further investigate the cytotoxic effects of FG88 mAbs on tumour cells, scanning electron microscopy (SEM) was used to evaluate FG88 mAbs-induced ultra-structural changes to HCT15 tumour cells (Figure 3.29). Characteristically, pronounced aggregation of HCT15 cells were observed after incubation with FG88 mAbs. Morphologically, the FG88 mAbs-treated cells displayed a loss of surface microvilli, with the formation of membrane blebs and surface wrinkles. Importantly, cell surface pore formation suggests a cell death mechanism reminiscent of oncosis. Heterogeneous

pore sizes with diameters ranging from 0.2 to 1µm were observed after 2 hr as well as 20 hr of mAbs incubation period.



	1	2	3	4	5	6	7	8
HCT15 DNA	+	-	-	-	+	+	+	+
Jurkat DNA	-	+	+	+	-	-	-	-
FG88.2	-	-	-	-	+	+	-	-
FG88.7	-	-	-	-	-	-	+	+
Anti-Fas	-	+	+	-	-	-	-	-
Z-FMK-VAD	-	-	+	-	-	+	-	+

Figure 3.28: DNA fragmentation analysis of FG88 mAbs-treated HCT15 cells. Agarose gel (0.8%) electrophoresis of DNA from HCT15 cells treated with FG88 mAbs in the presence (lane 6 and lane 8) and absence (lane 5 and lane 7) of 20 µM Z-FMK-VAD. Jurkat cells treated with anti-Fas mAb in the presence (lane 3) and absence (lane 2) of Z-FMK-VAD was included as positive and negative control respectively. Untreated HCT15 and Jurkat cells were included as negative controls (lanes 1 and 4, respectively).

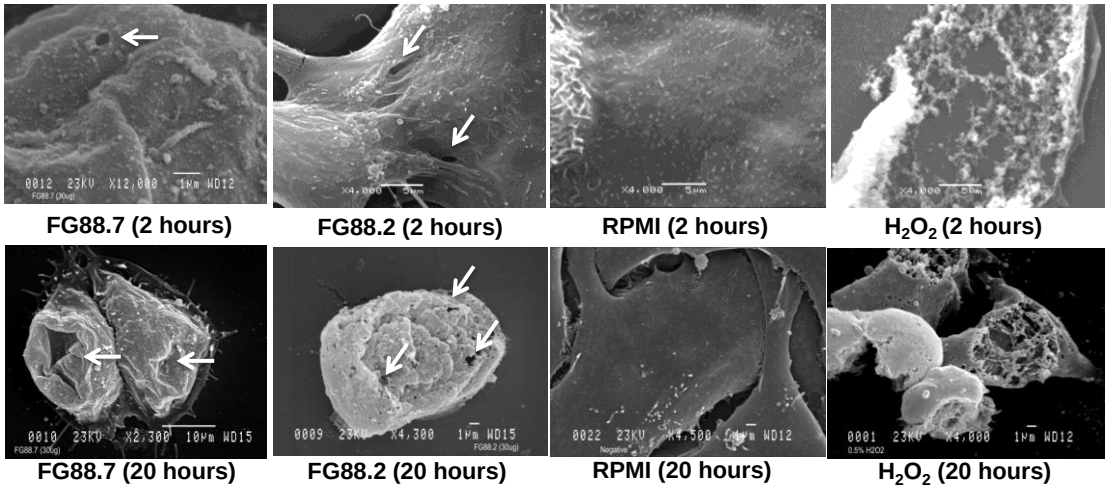


Figure 3.29: SEM analysis of FG88 mAbs-induced ultrastructural cellular changes. HCT15 cells were treated with FG88 mAbs (2.2 x 10^{-7} M) for 2 or 20 hr. Medium alone (RPMI) and 0.5% (v/v) hydrogen peroxide (H_2O_2) were used as negative and positive controls respectively. Arrows indicated mAb-induced pores.

3.2.4.2: FG88 mAbs internalise

To assess whether FG88 mAbs have the potential to be conjugated to cytotoxic drugs and used as antibody drug conjugates (ADCs), FG88 mAbs were labelled with Alexa Fluor 488 and the labelling

efficiency was checked by flow cytometry analysis (Figure 3.30). Both FG88.2 and FG88.7 mAbs were labelled successfully and showed binding to HCT15 cells.

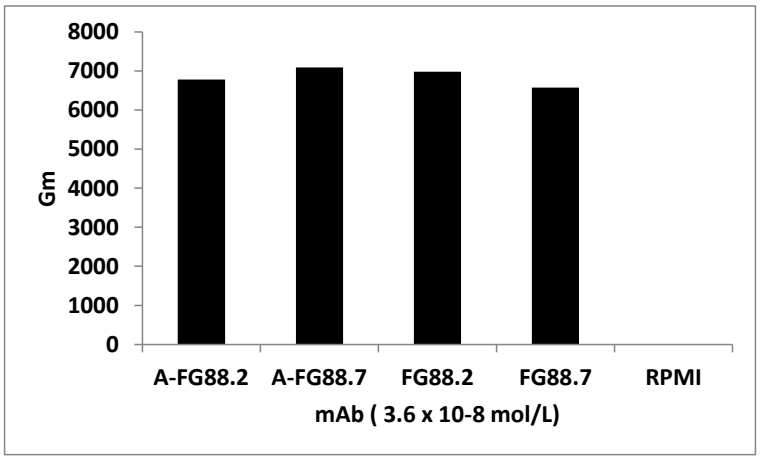


Figure 3.30: Binding of labelled FG88 mAbs to HCT15 cells by flow cytometry analysis. Alexa Fluor 488-labeled-FG88.2 (A-FG88.2) and FG88.7 (A-FG88.7) mAbs bound to HCT15 cells compared to the unlabelled mAbs. Medium alone (RPMI) was used as negative control.

Next, confocal microscopy was used to study Alexa Fluor 488-labelled FG88 mAbs incubated with HCT15 cells over a 2 hr period, showing internalisation of a proportion of mAbs (Figure 3.31). In addition, the FG88 membrane-staining pattern suggested heterogeneous distribution of the glycoepitope in the HCT15 plasma membrane. Over time, internalised FG88 mAbs co-localised with lysosomal compartments. Importantly, internalisation was validated through toxicity of Fab-ZAP-FG88 immune complexes containing saporin (Kohls & Lappi, 2000). Internalisation of the Fab-ZAP-FG88.2 and Fab-ZAP-FG88.7 complexes, but not the Fab-ZAP alone, led to dose dependent (IC_{50} 10^{-11} M) decrease in cell viability of the high glycoepitope expressing HCT15 cells (Figure 3.32). The moderately (HT29) binding cells were more refractory to the antibody internalisation.

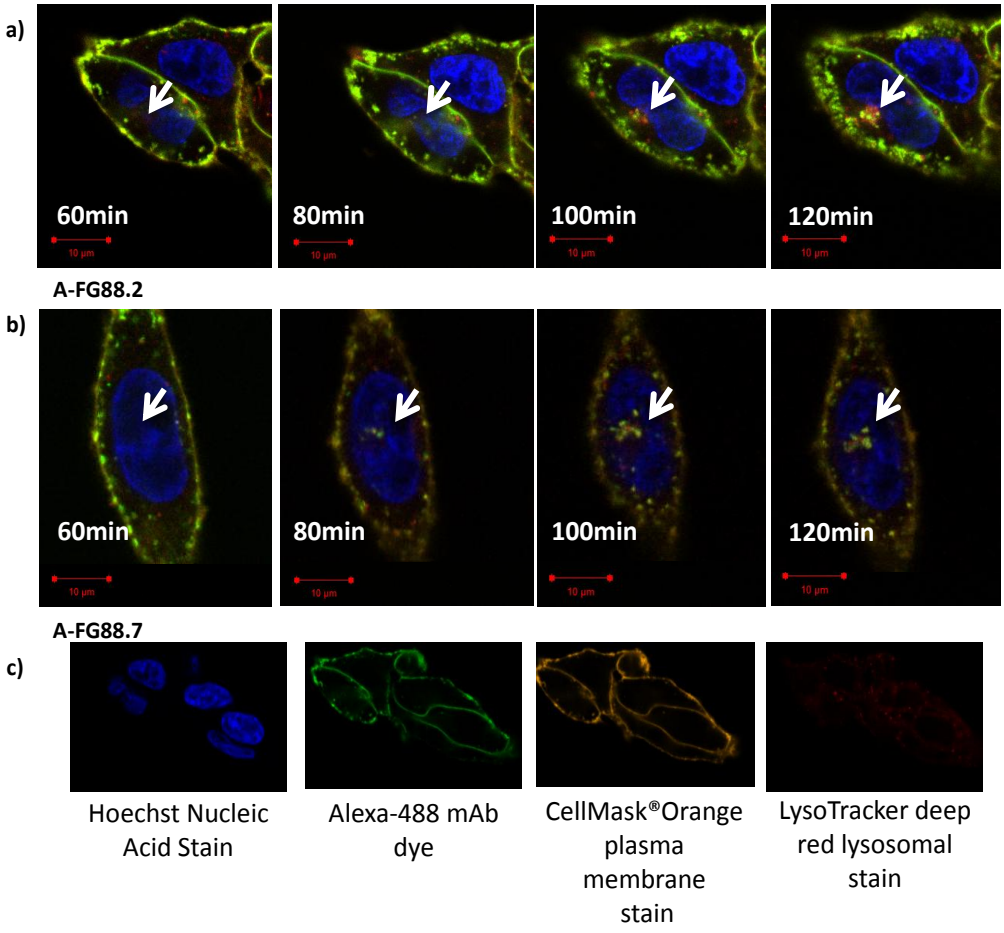


Figure 3.31: Time-lapse confocal microscopy of Alexa Fluor 488-labelled FG88 mAbs internalising into live HCT15 cells. Merged images taken every 20 min demonstrate a) A-FG88.2; b) A-FG88.7 mAbs internalisation and co-localisation with lysosomal compartments (arrows). c) The nucleus was labelled with Hoechst 33258, lysosomal compartments with LysoTracker Deep Red and the plasma membrane with CellMask Orange (*magnification x60*).

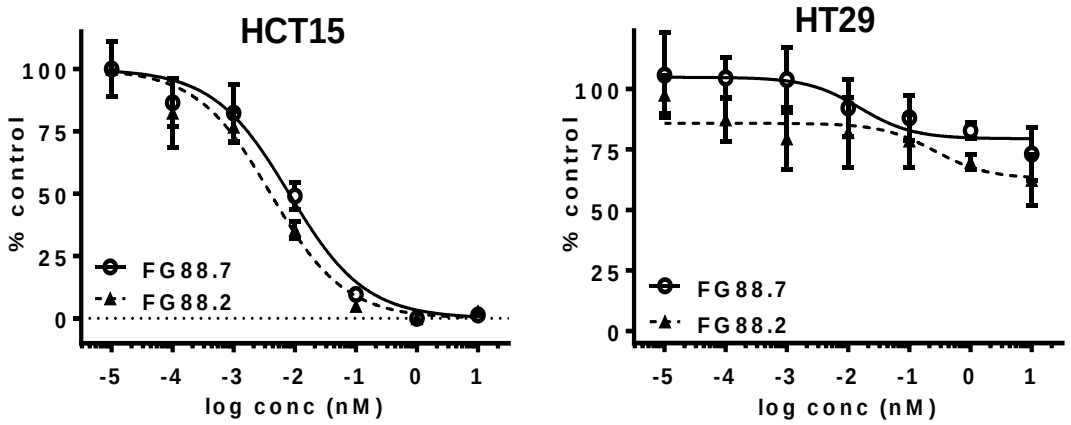


Figure 3.32: Reduction in the proliferation of two cancer cell lines (HCT15 and HT29) caused by a targeted toxin (saporin) delivered by internalised FG88 mAbs. The anti-proliferative effect of internalised FG88 mAbs preincubated with a saporin-linked anti-mouse IgG Fab fragment was

evaluated using ^{3}H-thymidine incorporation. Concentrations refer to FG88 mAbs. Results (mean ± STD from three independent experiments) are presented as a percentage of the proliferation of cells treated with primary mAbs only; normalised values are shown for HCT15. Non-linear regression was performed using GraphPad Prism 6.

3.4.2.3: FG88 mAbs exhibit excellent immune mediated cancer cell killing (ADCC and CDC) in vitro

The ability of the FG88 mAbs to induce Colo205 tumour cell death in the presence of human PBMCs through ADCC was investigated. Both FG88 mAbs induced potent cell lysis of the high-binding Colo205 cells in a concentration-dependent manner with EC_{50} values of 10^{-9} M and near 100% lysis at 7.4 x 10^{-9} M (Figure 3.33a). Next, a range of tumour cell lines were analysed for their susceptibility to FG88-mediated ADCC. The FG88 mAbs significantly lysed the high glycoepitope-expressing Colo205 and HCT15 above the killing observed with PBMCs alone (Figure 3.33b). The mAb 791T/36, showed no significant killing over the background observed with PBMCs alone, in the majority of cell lines. PBMC killing in the absence of FG88 mAbs was highest for cell lines lacking MHC-I, such as HCT15 and AGS, and probably reflects NK killing. Noticeably, less or no immune-mediated killing was seen with the FG88 mAbs on the moderate binding (HT29) and negative binding (OVCAR3 and AGS) cells even at high mAb concentration 6.7 x 10^{-8} M.

In addition, the FG88 mAbs were very efficient at inducing CDC of high binding HCT15 cells in the presence of human complement with nanomolar EC_{50} and over 80% lysis at 7.4 x 10^{-9} M mAb (Figure 3.33c). The FG88 mAbs displayed significant CDC activity against Colo205 cells and to a lesser degree DMS79 cells (Figure 3.33d).

In order to relate tumour staining by IHC to the antigen density requirement for cell killing, HCT15 xenograft tumour tissue was stained with FG88.2. From this analysis, the antigen density required for cell killing by FG88 mAbs was comparable with a score of 2 to 3 (moderate to strong) in the IHC analysis of the tumour tissues and accounts for 39% to 53% of gastrointestinal cancers (Table 3.5). Some of the normal gastrointestinal tissues also showed weak to moderate staining but this is mainly apical cells and it is unclear if mAb would be accessible to these cells in a therapeutic setting.

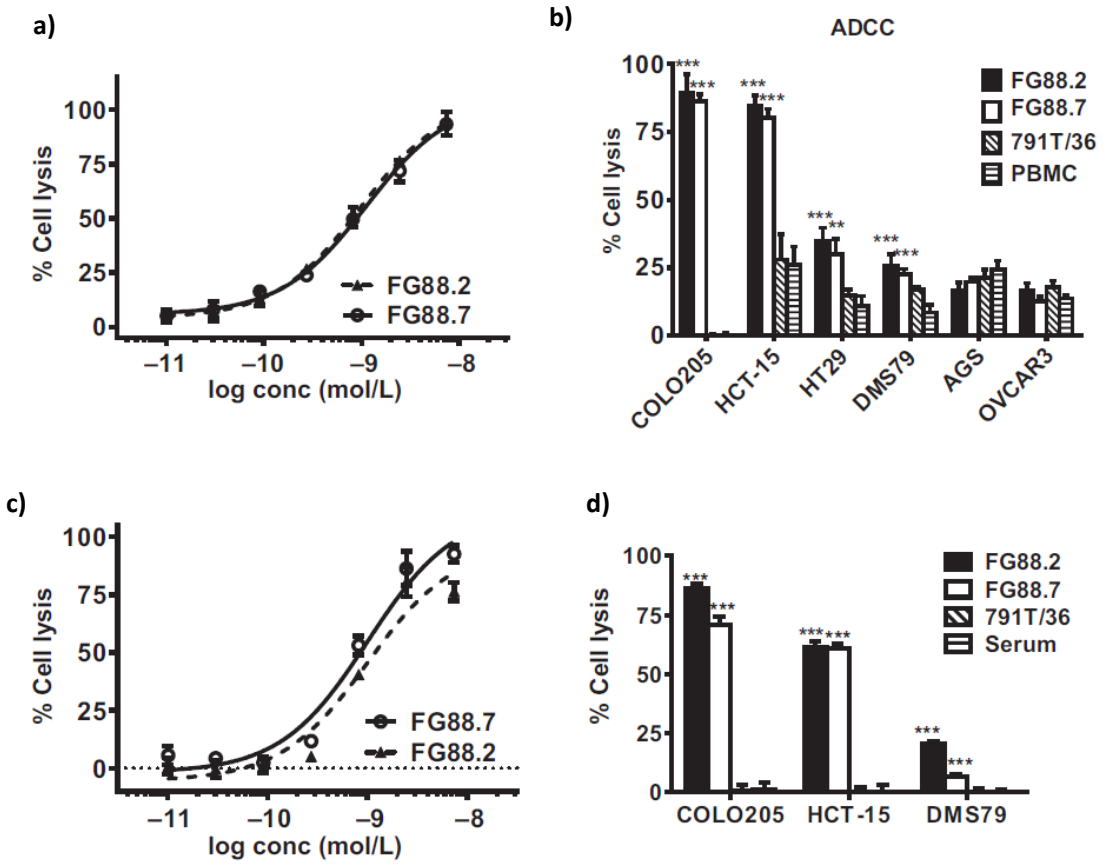


Figure 3.33: In vitro immune effector activity of FG88 mAbs. a) dose-dependent ADCC activity of FG88 mAbs on Colo205 cells. ^{51}Cr-labeled Colo205 cells were co-incubated with increasing concentrations of FG88 mAbs and human PBMCs (E:T of 100:1). b) ADCC activity of FG88 mAbs on a panel of tumour cell lines. FG88 mAbs and control (791T/36) were used at 7.3 x 10^{-8} M. c) dose-dependent CDC activity of FG88 mAbs on HCT15 cells. The ^{51}Cr-labeled HCT15 cells were incubated in the presence of increasing concentrations of FG88 mAbs in the presence of human serum. d) CDC activity of FG88 mAbs on a panel of tumour cell lines. FG88 mAbs and the negative control (791T/36) were used at 7.3 x 10^{-8} M. Significance (b and d) versus PBMC or serum control, respectively, was established by ANOVA followed by the Bonferroni multiple comparisons test, GraphPad Prism 6 (**, P< 0.01; ***, P< 0.001 versus control).

3.2.5: Potent *in vivo* anti-tumour activity by the FG88 mAbs in a human hepatic metastasis xenograft model (by Phil Clarke, University of Nottingham)

The mouse HCT15HM2 DLuX human hepatic metastasis tumour model was used to investigate the anti-tumour activity of the FG88 mAbs. The HCT15HM2 DLuX cell line is a bioluminescent variant of a liver metastasizing HCT15 cell line. Carcinoma establishment, growth and metastasis were assessed non-invasively via optical imaging. FG88 mAb treatment was initiated 10 days following tumour cell implantation and metastasis to the liver. The mAbs (100µg) or vehicle (PBS) was administered i.v. twice weekly, for 120 days. FG88.2 and FG88.7 significantly reduced tumour growth compared with vehicle control, by day 59 (P = 0.016; Mann-Whitney U test) and day 65 (P = 0.046; Mann-Whitney U

test), respectively (Figure 3.34a, b). FG88.7 mAb treatment led to a significant survival benefit [*P* = 0.0037; HR, 3.06; 95% confidence interval (95% CI), 2.26-17.39, log rank test] compared to vehicle alone (Figure 3.34c).

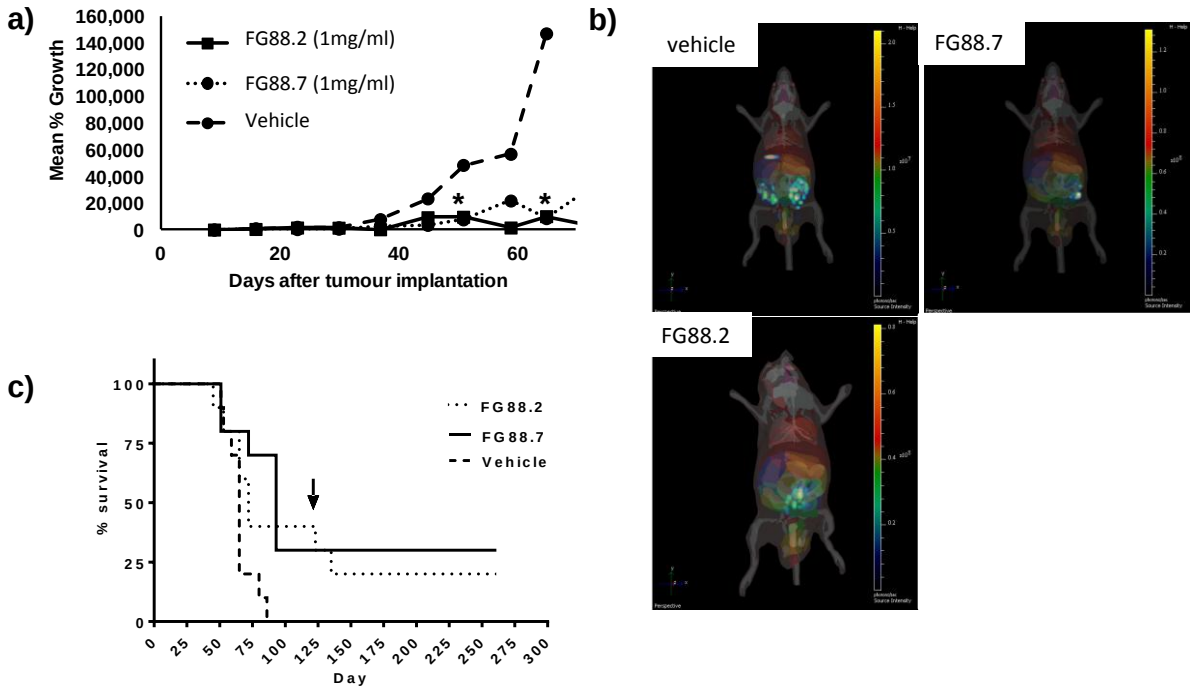


Figure 3.34: In vivo activity of FG88 mAbs in a mouse colorectal hepatic metastasis model. a) Reduction in percentage tumour growth (ventral bioluminescent signal) by FG88 mAbs. FG88.2 and FG88.7 significantly reduced tumour growth by day 59 (*P* = 0.016) and day 65 (*P* = 0.046), respectively, compared with the vehicle (PBS) control (Mann-Whitney U test). b) Bioluminescent imaging data at day 59 showing both FG88.2 and FG88.7 mAbs reduced tumour volume compared to vehicle control. c) Survival benefits due to anti-tumour activity of FG88.2 and FG88.7 mAbs. The HCT15HM2 DLuX mouse xenograft model was used to compare the anti-tumour effect of the FG88 mAbs to vehicle control (group size n> 8). MF-1 nude mice received mAbs (100µg) i.v. biweekly, treatment was initiated 10 days after tumour inoculation and the final dose was administered on day 120 (arrow).

3.5: Discussion

In this study, the production and characterisation of two novel murine anti-glycolipid IgG3 mAbs designated FG88.2 and FG88.7 was described. The glycome analysis of binding to over 610 sugars showed that the overall glycol-reactivity of both mAbs was similar. Both mAbs showed a preference for extended type I chain non-sialylated Le^a-containing carbohydrates and Le^cLe^x (Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAc) the later has not previously been described as a target for cancer therapy.

Lewis (Le) carbohydrate antigens are formed by the sequential addition of fucose onto oligosaccharide precursor chains on glycoproteins and glycolipids through the concerted action of a set of glycosyltransferases (Stanley and Cummings, 2009). Type I chains (containing Galβ(1→3)GlcNAc) for Le^a and Le^b, whereas type II chains (containing Galβ(1→4)GlcNAc) for Le^x and Le^y. Le^a and Le^b antigens are regarded as blood group antigens, yet many human cancers express Le^a or Le^b antigens regardless of Lewis blood group status (S. Hakomori & Andrews, 1970; H. Inagaki, Sakamoto, Nakazato, Bishop, & Yura, 1990; J. Sakamoto et al., 1986). In addition, Le^a and Le^b antigen frequently coexist in human tumour cells (Blaszczyk, Pak, Herlyn, Sears, & Steplewski, 1985). Dimeric Le^a and Le^x have also been identified as tumour associated antigens in breast and gastrointestinal carcinomas (Cazet et al., 2010; H. Inagaki et al., 1990; J. Sakamoto et al., 1986).

Circulating Le^a- and Le^b-containing glycolipids can be adsorbed by erythrocytes (Jewett & Tseng, 2011) depending on the secretor status of individuals. The term ABH secretor refers secretion of ABO blood group antigens in body fluids (D'Adamo & Kelly, 2001). If the antigen is derived from both *Le* and *Se* alleles, Le^a is converted to Le^b, which can be absorbed to erythrocyte membrane, resulting in Le^{a-b+} phenotype. If the antigen derives from only *Le* allele, the antigenic form of Le^a will be expressed, giving rise to the Le^{a+b-} phenotype. If Lewis antigen does not carry the *Le* allele, regardless of the presence or absence of *Se* allele, the erythrocyte phenotype will be Le^{a-b-}(M. J. Kim et al., 2002). Among Lewis antigen positive individuals, ABH secretors are always Le^{a-b+} whereas ABH non-secretors are always Le^{a+b-}(D'Adamo & Kelly, 2001). In Caucasians, it was reported that approximately 80% are secretor and 20% are non-secretor. Importantly, FG88 mAbs have no reactivity with erythrocytes from Le^a-positive human donors, suggesting that FG88 mAbs preference for more complex Le^a-containing glycans precludes erythrocyte reactivity.

Le^x is found to be expressed abundantly in breast and gastrointestinal carcinomas as well as chronic myeloid leukemia. Normal expression of Le^x is restricted on normal human polymorphonuclear neutrophils (PMNs). In this study, we have shown that FG88.2 and FG88.7 did not bind to any blood cells including granulocytes (neutrophils, basophils and eosinophils) and peripheral blood mononuclear cells (PBMCs; lymphocytes, NK cells and monocytes). This is important because in a phase I clinical trial utilising FC-215 (IgM, murine anti- Le^x mAb) on 11 patients with advanced cancers, although anti-tumour responses were seen, the mAb caused transient but profound neutropenia (Ballare et al., 1998; Capurro et al., 1999). Therefore, although FG88 mAbs recognise Le^x, it is unlikely that they would induce neutropenia.

By relating antigen density by IHC to the density required for cell killing, it was observed that tumours needed to stain moderately to strongly to be targets for FG88.2. This would include 53% of

gastrointestinal cancers and 5% of breast cancers. Importantly, the significant association of strong FG88.2 binding with poor outcome in colorectal and breast cohorts, independent of stage and vascular invasion in colorectal cohort, suggests that the most aggressive colorectal and breast cancers would be most likely to benefit from FG88.2 therapy. Earlier work has demonstrated the prognostic value of Le^x, SLe^x and SLe^a expression in colorectal and breast carcinomas and the association of SLe^a and SLe^x expression with increased metastatic potential in colorectal and breast cancer respectively(Brooks & Leathem, 1995; Y. Inagaki et al., 2014; Julien et al., 2011; Kannagi, 2007; Koh, Lee, Ahn, Lee, & Gong, 2013; Nakagoe et al., 2000; Weston et al., 1999), but this study is the first to demonstrate independent prognostic value for Le^cLe^x expression in colorectal cancer as defined by FG88.2 mAb.

FG88.2 is significantly associated with the lack of ER, PgR and HER2 expression, expression of basal markers (CK5/6 and/or CK14 and/or EGFR) and both triple negative and basal like phenotype. This promising result suggested that FG88.2 could be useful as first line and second line treatment of triple negative basal like breast cancer subtypes and endocrine resistance ER-positive breast cancer respectively.

Some of the normal gastrointestinal tissues also showed weak to moderate staining with FG88.2, but this was mainly to apical cells and it is unclear whether mAb will be accessible to these cells in a clinical setting. Indeed, SC104 mAb showed similar staining but showed no toxicity in primate models and no toxicity in clinical trials (NCT01447732, SC104/CEP-37250/KHK2804) have been reported. Lewis antigens are only synthesised by primates due to FUT3/4 expression. In this context, it was of interest that there was a significant association between FG88.2 binding and FUT3 expression in the colorectal cancer cohort. Formal toxicity studies would thus need to be done in cynomolgus monkeys. Staining of a cynomolgus monkey normal TMA with FG88.2 showed a similar binding pattern to that observed in normal human tissues.

The FG88 mAbs exhibited potent direct growth-inhibitory effect ($IC_{50} \sim 10^{-8}$ M) on high glycoepitope-expressing HCT15 and Colo205 colorectal tumour cells but not on low or moderately expressing tumour cells, suggesting a contribution of the high functional affinity with which the FG88 mAbs bind to tumour cell surfaces. The lack of cell killing of low to moderate glycoepitope-expressing cells offers a further protection for normal tissues. The growth inhibitory effect of the FG88 mAbs was characterised by cell aggregation followed by an effect on cell membrane integrity and pore formation. More detailed characterisation of the cytotoxic effect revealed absence of DNA fragmentation and limited involvement of effector caspases, suggesting a non-apoptotic mechanism. A number of other anti-glycan mAbs have been shown to induce non-apoptotic oncosis-like cell

death (Hernandez et al., 2011; Loo et al., 2007; H. L. Tan, Fong, Lee, Yap, & Choo, 2009; S. Zhang et al., 1998). Anti-CD20 mAbs in the treatment of B cell lymphoma have been classified functionally in type I and II mAbs. Type II anti-CD20 mAbs, such as tositumomab, are effective in mediating direct cell killing in a caspase independent manner, which was associated with homotypic cell adhesion and exhibits many similarities with the FG88 mAbs direct cell killing (Alduaij et al., 2011). In both cases, release of cellular content into the extracellular space via mAb-induced pore formation, may constitute a form of immunogenic cancer cell death (ICD) through the release of danger-associated molecular patterns ['DAMPS'; (Krysko, Love Aaes, Bachert, Vandenabeele, & Krysko, 2013)]. This ICD may reinitiate immune responses in the immunosuppressive tumour microenvironment. In contrast, SC104, which recognises sialylated Le^a-related glycans, was also cytotoxic to high binding cells, such as HCT15 and Colo205, but with a higher IC_{50} (2×10^{-7} M) and via classical apoptosis with evidence of caspase activation (Durrant et al., 2006).

Two independent approaches demonstrated efficient internalisation and lysosomal localisation of a proportion of the FG88 mAbs in a glycoepitope density-dependent manner. This indicates their potential clinical usefulness for ADC development. Furthermore, a fraction of the FG88 mAbs remains at the cell surface where their slow dissociation kinetics enables excellent immune-effector functions (ADCC and CDC). Importantly, moderate to weak binding cells were less susceptible to ADCC and CDC, offering a further level of protection for normal cells expressing low levels of the antigen.

Carbohydrate-recognising mAbs generally exhibit weak glycan affinity. In contrast, the FG88 mAbs displayed strong functional affinity for a high-density Le^a-HSA glycoconjugate but nanomolar functional affinity for tumour cell lines overexpressing the Le^a-containing glycoepitope such as Colo205 and HCT15. This probably reflects the more complex binding behaviour of the mAbs at the cell surface where the mixed glycoepitope (glycolipid and glycoprotein) recognition, the fluid membrane, or the potential for internalisation and cell killing can explain the higher cell-surface K_d.

The excellent *in vitro* cytotoxicity of both mAbs translated into potent anti-tumour efficacy and significant survival improvement in a colorectal hepatic metastasis xenograft model in which mAb treatment was initiated 10 days after tumour initiation and development of liver metastasis. The FG88 mAbs cured both intraperitoneal disease and liver metastases in 30% of animals. Post-mortem imaging analysis of those animals showed very limited evidence of tumour regrowth. Previous xenograft studies have shown good anti-tumour efficacy when treatment was initiated at the time of tumour implantation or shortly afterwards (Schreiber, Hellstrom, & Hellstrom, 1992; Watanabe et al., 1991). No studies, to date, have shown tumour eradication of 10-day established tumours and

liver metastases. SC104 inhibited tumour growth *in vivo*, but was more effective in the tumour prevention model and only worked in the therapeutic models in combination with 5-FU/leucovorin (Durrant et al., 2006). The humanised SC104 (NCT01447732) currently in phase I/II trials (SC104/CEP-37250/KHK2804) has been defucosylated for enhanced effector functions. Further studies have been initiated to evaluate the therapeutic potential of human IgG1 chimeric versions of the FG88 mAbs and these results will be presented in the next chapter.

Chapter 4: E345R mutation restores direct killing function in chimerised mIgG3

4.1 Background

In the previous chapter a high affinity, internalising, parental murine FG88.2 mAb (IgG3) was shown to bind specifically to Le^cLe^x-related glycans. FG88.2 mAb exhibited potent immune effector functions such as ADCC and CDC as well as direct cytotoxicity via a caspase independent mechanism. The limitation of the parental murine FG88.2 mAb as a therapeutic is the induction of a human anti-mouse immunoglobulin immune response in patients (Gorman & Clark, 1990). To overcome this, FG88.2 mAb was chimerised to generate CH88.2 mAb which contains the human constant regions with murine variable regions. In this chapter we show that, the chimeric mAb bound well and a percentage was rapidly internalised, suggesting that it could be a good candidate for ADC; however, it lost its direct killing ability.

Recently, it was demonstrated that IgG can form surface clusters through Fc-dependent interactions to form hexamers, which in turn leads to very efficient C1q activation, and that the E345R mutation enhanced antibody hexamer formation for all IgG isotype variants (Diebolder et al., 2014). The recently described structure of the deglycosylated IgG4 Fc further supports this hypothesis of a possible generic hexameric Fc assembly, which is stabilised by this E345R mutation (A. M. Davies, Jefferis, & Sutton, 2014).

In order to determine if the E345R mutation enhanced CH88.2 binding or killing, the single amino acid substitution at position E345R (Glu^{345}→ Arg) was introduced into CH88.2 to generate a mutant version of CH88.2 named ECH88.2 mAb.

Mutating E345R restored the direct killing of tumour cells and improved CDC activity of the mAb. Furthermore, ECH88.2 showed strong tumour reactivity suggesting its potential as a stand-alone therapeutic mAb.

4.2: Results

4.2.1: Antigen binding and biological activities of the chimeric version of FG88.2 (CH88.2) mAb

In order to reduce the immunogenicity of the parental murine FG88.2 mAb, a chimeric version (CH88.2) was generated by Dr Richard McIntosh by PCR amplification of the heavy chain and kappa chain variable regions and cloning into a dual expression vector in-frame with pre-existing human constant regions. The antigen binding, direct cytotoxicity, ADCC, CDC and internalising activities of the CH88.2 mAb were measured (Figure 4.1). The CH88.2 mAb showed reduced antigen binding activity to Colo205 cells compared to FG88.2 mAb (FG88.2 Gm: 2049; CH88.2 Gm: 995; Figure 4.1a) by flow cytometry and had a greatly reduced, although still significant, ability to mediate CDC on HCT15 cells at 2.4 x 10^{-8} M (maximum killing of 65% by FG88.2 and 48% by CH88.2; Figure 4.1b). In contrast, CH88.2 mAb showed an improvement in inducing ADCC compared to FG88.2 mAb (IC_{50} FG88.2: 1 x 10^{-9} M; IC_{50} CH88.2: 1.2 x 10^{-10} M; Figure 4.1b). Unlike FG88.2 mAb, CH88.2 mAb was unable to induce direct tumour cell death as determined by a PI uptake assay (Figure 4.1c). In order to determine the ability of the CH88.2 mAb to internalise into tumour cells, CH88.2 mAb was labelled with Alexa Fluor-488 dye (A-CH88.2) and flow cytometry analysis was used to confirm successful labelling and binding of the labelled mAb to Le^c-Le^x positive HCT15 cells (data not shown). The uptake of A-CH88.2 mAb by HCT15 cells was studied by confocal microscopy. As shown in Figure 4.1d, A-CH88.2 mAb binding to HCT15 cells over a two-hour period efficiently internalised and co-localised with lysosomal compartments.

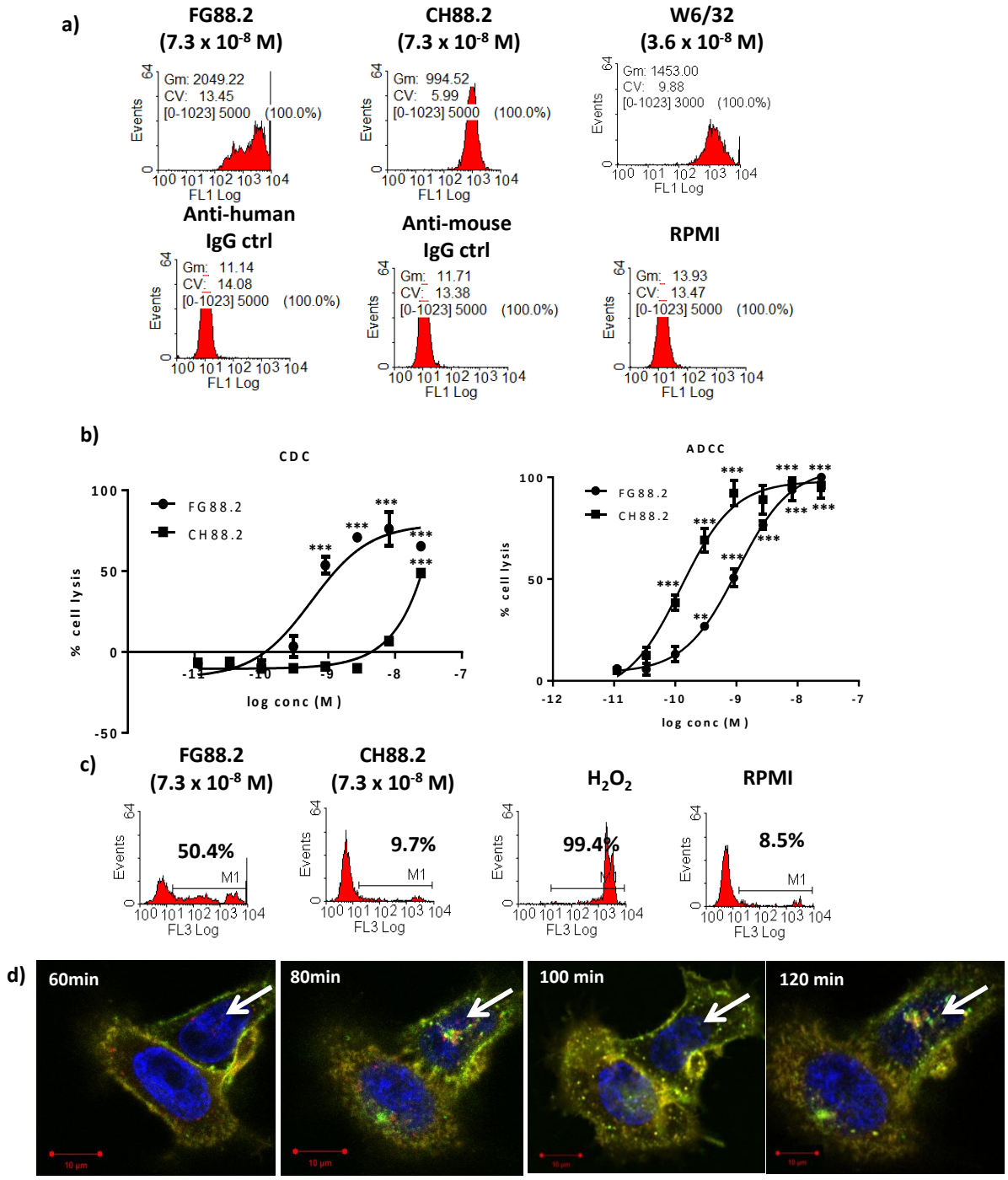


Figure 4.1: Antigen binding and biological activities of CH88.2 mAb compared to the murine FG88.2 parental mAb. a) Binding of CH88.2 to Colo205 cells was assessed by indirect immunofluorescence and flow cytometric analysis. Parental FG88.2 mAb and W6/32 antibody were included as positive controls. Secondary anti-human and anti-mouse immunoglobulins and medium alone (RPMI) were negative controls. b) Dose dependent CDC (^{51}Cr-labeled cells were incubated in the presence of increasing concentrations of CH88.2 mAb in the presence of human serum) and ADCC [^{51}Cr-labeled cells were co-incubated with increasing concentrations of CH88.2 mAb and human PBMCs (E:T of 100:1)] activity of CH88.2 mAb compared to FG88.2 mAb on Colo205 cells. Significance versus serum (CDC) or PBMC (ADCC) control, respectively, was established by ANOVA followed by the Bonferroni multiple comparisons test, GraphPad Prism 6. c) Direct cytotoxicity activity of CH88.2 mAb measured

by PI uptake and flow cytometric analysis on Colo205 cells incubated with CH88.2 at 7.3 x 10^{-8} M. FG88.2 mAb was included as comparison, hydrogen peroxide (H_2O_2) and medium alone (RPMI) were positive and negative control respectively. d) Time-lapse confocal microscopy of Alexa-Fluor-488-labelled CH88.2 (A-CH88.2) mAb internalising into live HCT15 cells. Merged images taken every 20 min demonstrate A-CH88.2 mAb internalisation and co-localisation with lysosomal compartments (arrows). The nucleus was labelled with Hoechst 33258, lysosomal compartments with LysoTracker Deep Red and the plasma membrane with Cell Mask Orange (*magnification x60*).

4.2.2: E345R mutation enhanced the cell surface antigen binding ability of ECH88.2 mAb

The E345R mutation, located 5 amino acid residues into human IgG1 C_H3, was introduced into CH88.2 mAb in order to improve CDC and direct killing (by convention, 'Kabat' numbering of the heavy chain constant region starts at residue 118; Dr Richard McIntosh). To assess whether the introduction of the E345R mutation to CH88.2 altered the cognate binding of the mAb to tumour cell surface antigens, FG88.2 (mIgG3), CH88.2 (hIgG1) and ECH88.2 (hIgG1 E345R) mAbs were biotinylated and the relative binding of these mAbs to HCT15 cells was assessed by indirect immunofluorescence and analysed by flow cytometric analysis as shown in Figure 4.2. All three mAbs demonstrated a specific, dose dependent increase in binding to tumour cell surface antigens. However, FG88.2 mAb (K_d ~ 4.9 x 10^{-8} M) showed better binding compared to CH88.2 and ECH88.2 mAbs despite the fact that they have identical V domains. ECH88.2 mAb (K_d ~ 1.9 x 10^{-7} M) showed similar binding to CH88.2 mAb (K_d ~ 2.6 x 10^{-7} M). Next, Le^a-HSA binding kinetics of ECH88 and CH88.2 mAbs were examined using SPR (Biacore 3000) (Dr Mireille Vankemmelbeke) and compared to FG88.2 mAb. In the previous chapter, it was demonstrated that the FG88.2 mAb has a strong functional affinity (K_d ~ 10^{-10} M) with fast association (k_{on}: ~ 10^5 1/Ms) and slow dissociation (k_{off}: ~10^{-5} 1/s) rates. The CH88.2 mAb showed weaker functional affinity (K_d ~ 5.9 x 10^{-8} M ± SD 9.2) with slower K_{on} ~ 10^4 1/Ms and faster K_{off} ~ 10^{-2} 1/s compared to the FG88.2 mAb. Introduction of the E345R mutation showed improvement in functional affinity, association and dissociation rate of ECH88.2 mAb (K_d ~ 0.9 x 10^{-8} M ± SD 0.4; K_{on} ~ 10^5 1/Ms; K_{off} ~10^{-4} 1/s) compared to the CH88.2 mAb.

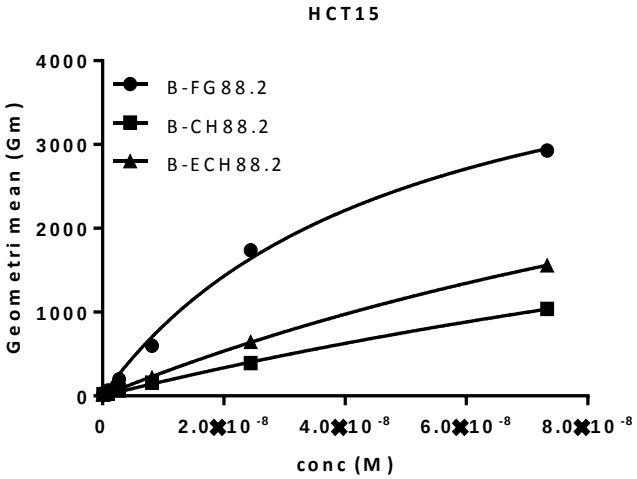


Figure 4.2: Binding of ECH88.2 and CH88.2 to HCT15 cells. Binding of CH88.2 and ECH88.2 to HCT15 cells was compared to FG88.2 as assessed by indirect immunofluorescence and flow cytometric analysis. FG88.2, CH88.2 and ECH88.2 mAbs were biotinylated and binding was detected with streptavidin-FITC. Biotinylated mAbs binding in the concentration range 3.3 x 10^{-11} to 7.3 x 10^{-8} M are presented as geometric means (Gm). One site specific binding model, prism 6.

4.2.3: High functional affinity of FG88.2 due to IgG3s 'intermolecular cooperativity'

To assess whether FG88.2 mAb had self-binding ability and whether ECH88.2 restored this ability, an antibody self-binding ELISA was performed (Figure 4.3). The FG88.2 mAb demonstrated a dose-dependent increase in binding to itself whereas CH88.2 and ECH88.2 mAbs did not show any binding in the absence of antigen.

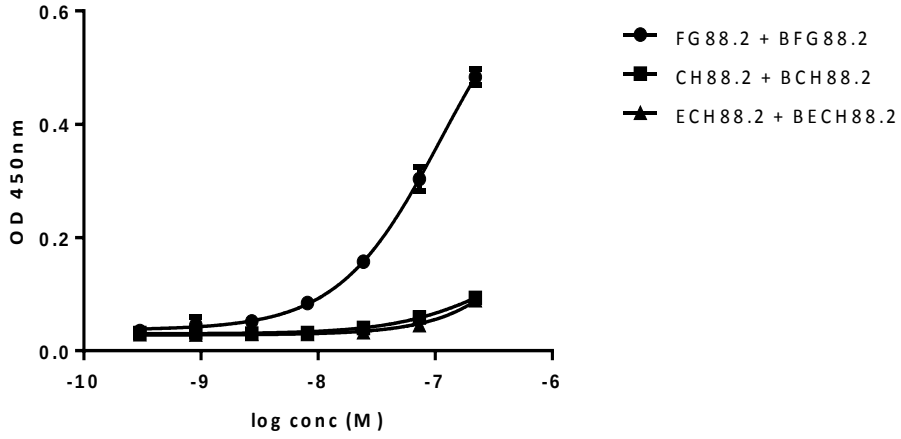


Figure 4.3: MAb self-binding ELISA. FG88.2, CH88.2 and ECH88.2 (200ng/well) were coated on 96 well plates overnight. A concentration range of corresponding biotinylated mAbs (BFG88.2, BCH88.2 and BECH88.2) were incubated in mAb-coated microwell plates.

Next, the ability of biotinylated FG88.2 mAb to bind to other murine IgG3 mAbs such as FG27.10 (anti-Le^y mAb), FG2811.72 (anti-SSEA3 mAb) and SC101 (anti-$Le^{y/b}$) was investigated. CH88.2 and ECH88.2 and PBS were included as negative controls (Figure 4.4). As shown in Figure 4.4, biotinylated FG88.2 bound to unlabelled murine IgG3 mAbs, FG88.2, FG27.10, FG2811.72 and SC101 but not to human IgG1 mAbs, consistent with the concept of the self-associating murine IgG3 mAbs.

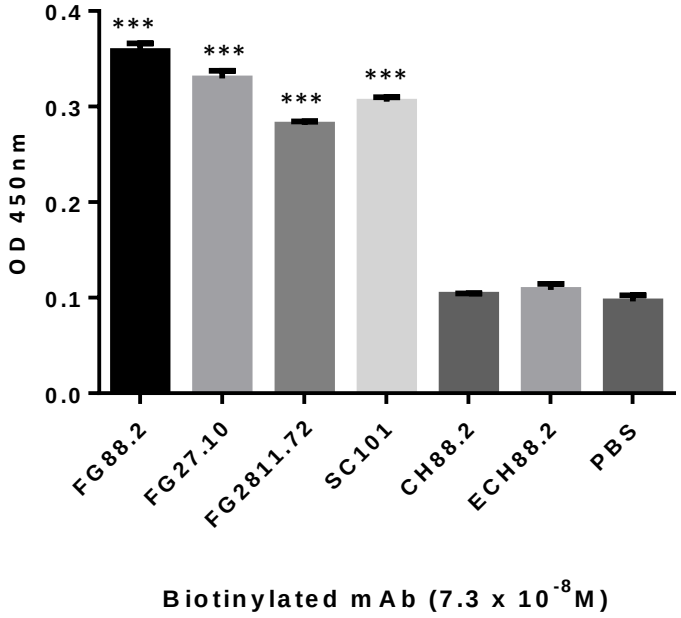


Figure 4.4: MAb self-binding ELISA. FG88.2 (200ng/50µl/well) was coated on 96 well plates overnight at 4°C. 50µl of a panel of biotinylated mAbs (7.3 x 10^{-8} M) were incubated in the FG88.2 mAb-coated microwells for 1 hr at room temperature. Binding between mAbs were detected with streptavidin-HRPO. Significance versus PBS, was established by ANOVA followed by the Bonferroni multiple comparisons test, GraphPad Prism 6. (***, P< 0.001 versus control).

4.2.4: E345R mutation improves CDC activity and maintains the ADCC ability of ECH88.2

Next, the enhancement of the ability of the ECH88.2 mAb in inducing CDC in the presence of human serum was investigated (Figure 4.5a). Colo205 cells served as target cells. FG88.2, CH88.2 and ECH88.2 showed a similar level of maximum tumour cell killing. FG88.2 had an IC_{50} of 7.3 x 10^{-11} M, CH88.2 an IC_{50} of 2.9 x 10^{-9} M and ECH88.2 an IC_{50} of 9.3 x 10^{-11}. However, within themAb concentration range from 1 x 10^{-10} to 2.4 x 10^{-8} M, ECH88.2 showed significant enhancement in CDC activity compared to CH88.2, suggesting the E345R mutation improved CDC activity of the ECH88.2 mAb. The ability of ECH88.2 to induce tumour cell death via ADCC was also screened (Figure 4.5b). Human PBMCs were used as the source of effector cells and Colo205 cells served as target cells. ECH88.2 also showed enhanced ADCC when compared to the parental mouse IgG3 FG88.2 and the

human IgG1 chimeric antibody FG88.2 (IC_{50}: 2.5 x 10^{-10} M), CH88.2 (IC_{50}: 5 x 10^{-11} M) and ECH88.2 (IC_{50}: 5.2 x 10^{-12} M).

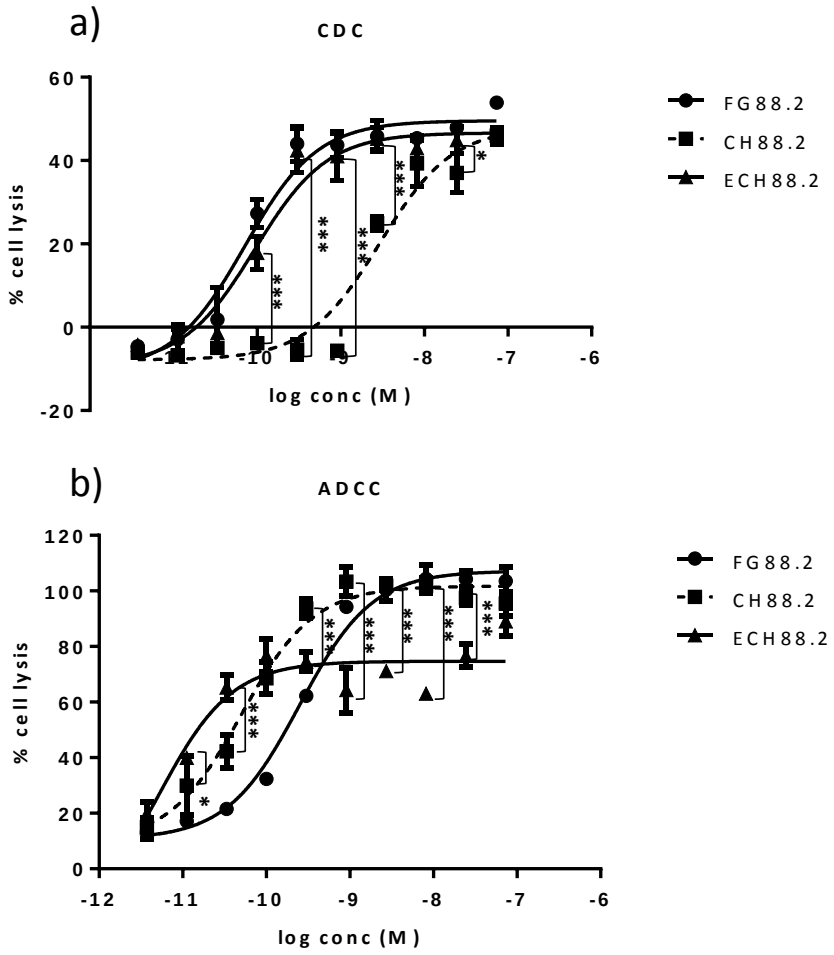


Figure 4.5: In vitro immune effector activity of ECH88.2 mAb. a) dose-dependent CDC activity of FG88.2, CH88.2 and ECH88.2 mAbs on Colo205 cells. The ^{51}Cr-labelled Colo205 cells were incubated with increasing concentrations of the mAbs in the presence of human serum. b) dose-dependent ADCC activity of FG88.2, CH88.2 and ECH88.2 mAbs on Colo205 cells. The ^{51}Cr-labelled Colo205 cells were incubated with increasing concentrations of the mAbs in the presence of human PBMCs. Significance between mAbs at a range of concentrations was established by two way ANOVA, GraphPad Prism 6.

4.2.5: The E345R mutation restores the ability of the human IgG1 CH88.2 mAb to induce PI uptake on tumour cells and inhibit tumour cell growth

The parental mouse IgG3, FG88.2 mAb exhibited potent direct cytotoxicity on tumour cells via caspase independent pathway (Chapter 3). However, after chimerisation, the human IgG1 CH88.2 mAb lost the ability to induce direct tumour cell death. To determine whether the E345R mutation restores the direct killing ability of ECH88.2 mAb, HCT15 and Colo205 cells were incubated with

ECH88.2 mAb at 7 x 10^{-9} M and 2 x 10^{-7} M and the percentage of PI uptake was measured (Figure 4.6). Both FG88.2 and ECH88.2 induced cell death at 7.3 x 10^{-8} M and 2.2 x 10^{-7} M, but CH88.2 did not. FG88.2 induced 63.9% of the HCT15 cells to take up PI at 7.3 x 10^{-8} M and 85.1% at 2.2 x 10^{-7} M; and 57.1% (7.3 x 10^{-8} M) and 82.7% (2.2 x 10^{-7} M) of Colo205 cells to take up PI. CH88.2 induced PI uptake in only 12.9% (7.3 x 10^{-8} M) and 21.2% (2.2 x 10^{-7} M) of HCT15 cells and 5.6% (7.3 x 10^{-8} M) and 5.7% (2.2 x 10^{-7} M) of Colo205 cells Whilst ECH88.2 induced 47% (7.3 x 10^{-8} M) and 76.3% (2.2 x 10^{-7} M) of HCT15 cells and 53.9% (7.3 x 10^{-8} M) and 83.8% (2.2 x 10^{-7} M) of Colo205 cells.

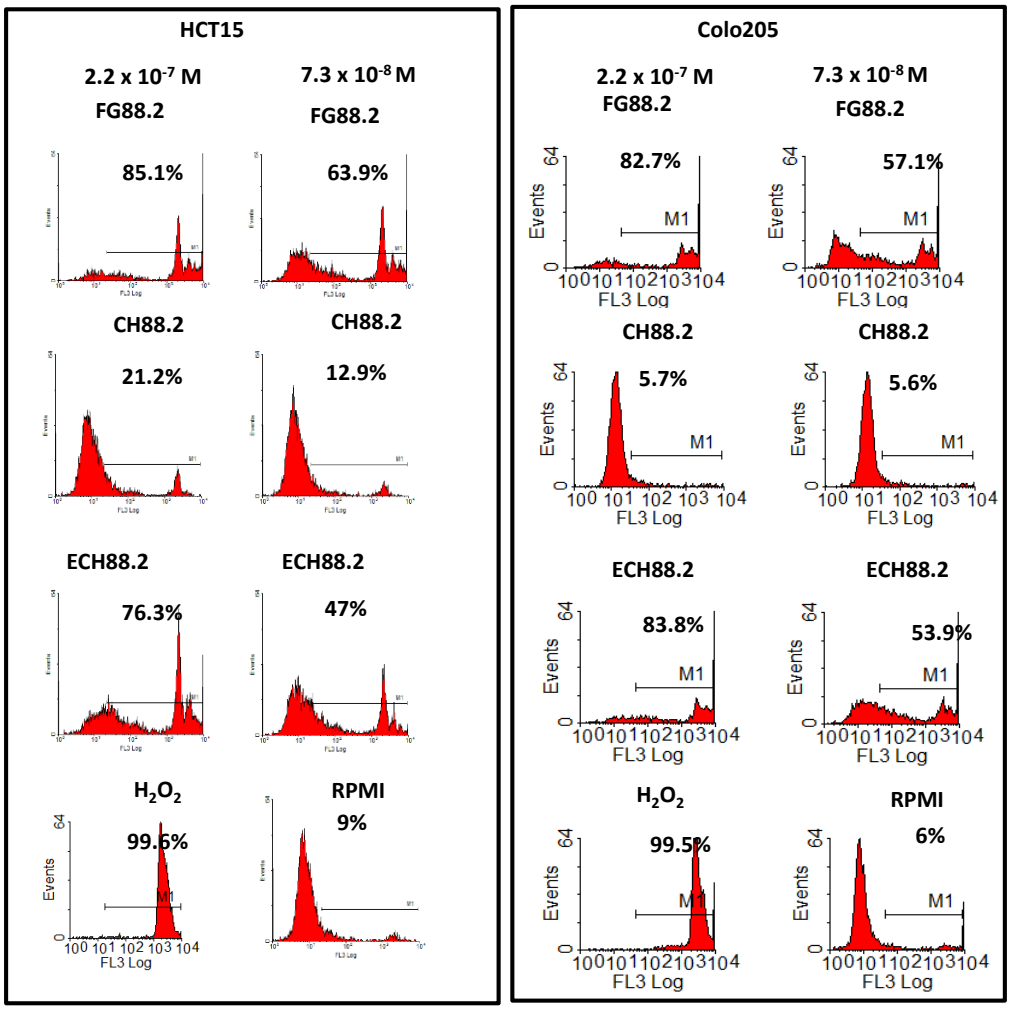


Figure 4.6: ECH88.2 induced direct cell death of HCT15 and Colo205 cells. HCT15 and Colo205 cells were incubated with 7.3 x 10^{-8} and 2.2 x 10^{-7} M of FG88.2, CH88.2 and ECH88.2 mAbs at 37°C and cell death was assessed by PI uptake and flow cytometric analysis. H_2O_2 and RPMI were the positive and negative controls respectively.

In addition, the effect of ECH88.2 mAb on the growth of HCT15 and Colo205 cells was determined using a WST-8 assay over a period of 96 hours (Figure 4.7). The growth of HCT15 and Colo205 cells were inhibited by approximately 80-85% by the addition of FG88.2 at 2.2 x 10^{-7} M with an IC_{50} of ~2.8

x 10^{-8} M for HCT15 and ~2.8 x 10^{-9} M for Colo205 after incubating with the mAb for 96 hr. CH88.2 mAb treatment had no effect on HCT15 cell growth and only approximately 30% inhibition on Colo205 cells at 2.2 x 10^{-7} M. ECH88.2 showed 67% inhibition of HCT15 growth and 82% of Colo205 cells at 2.2 x 10^{-7} M with an IC_{50} of ~1.9 x 10^{-7} M for HCT15 and ~ 2.3 x 10^{-8} M for Colo205, suggesting the E345R mutation partly restored the direct killing ability of the mAb. Light microscopy evaluation of FG88.2 and ECH88.2 treated Colo205 cells after 72 hr showed evidence of cell death, but this was not observed in the CH88.2-treated cells (Figure 4.8).

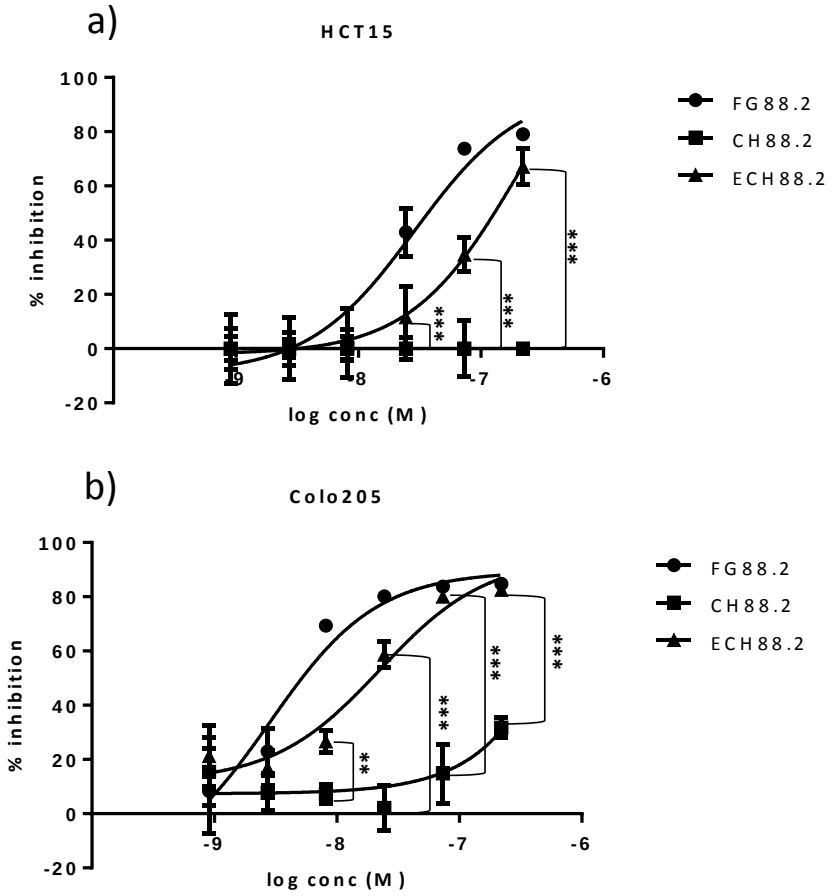


Figure 4.7: Direct cytotoxicity of ECH88.2 mAb on HCT15 and Colo205 cells. The anti-proliferative effect of ECH88.2 mAb was analysed using the WST-8 assay. a) HCT15 and b) Colo205 cells were incubated with mAb in a range of concentrations for 96 hr after which WST-8 reagent was added for the final 4 hr. Results are expressed as percentage inhibition compared to control cells and are representative of at least two independent experiments. Significance between mAbs with a range of concentrations was established by two way ANOVA, GraphPad Prism 6.

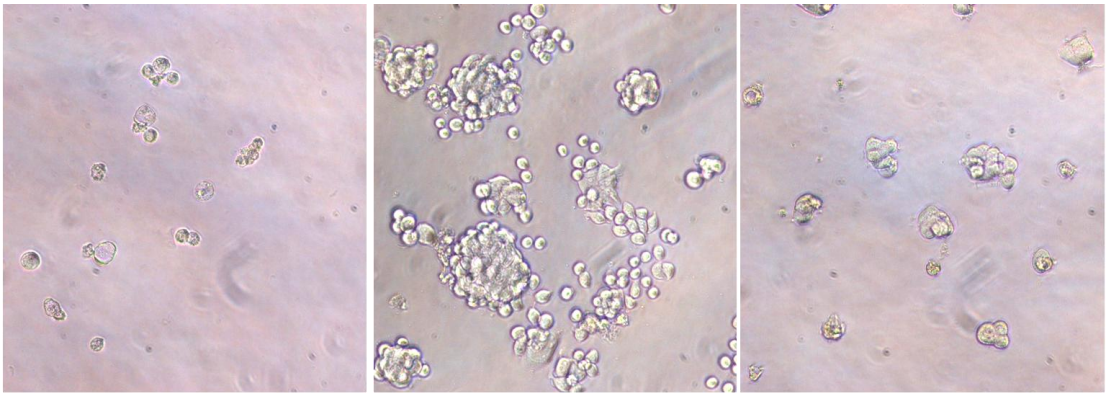


Figure 4.8: Phase-contrast imaging of ECH88.2-treated Colo205 cells. Phase-contrast light microscopy imaging showing Colo205 cells after incubation with ECH88.2 mAb at 2.2 x 10^{-7} M for 72 hr. FG88.2 and CH88.2 mAbs were included as comparisons (*magnification x 10*). Images were taken using a Nikon Eclipse TS100.

4.2.6: The ECH88.2 mAb internalises

To ensure that the E345R mutation did not affect the internalisation ability of the mAb, internalisation was validated through toxicity of Fab-ZAP-CH88.2 and Fab-ZAP-ECH88.2 immune complexes containing saporin (Figure 4.9). Internalisation of the Fab-ZAP-CH88.2 and Fab-ZAP ECH88.2 complexes, but not the Fab-ZAP alone or the Fab-ZAP pre-incubated with a control mAb, led to a dose-dependent decrease in cell viability of HCT15 cells.

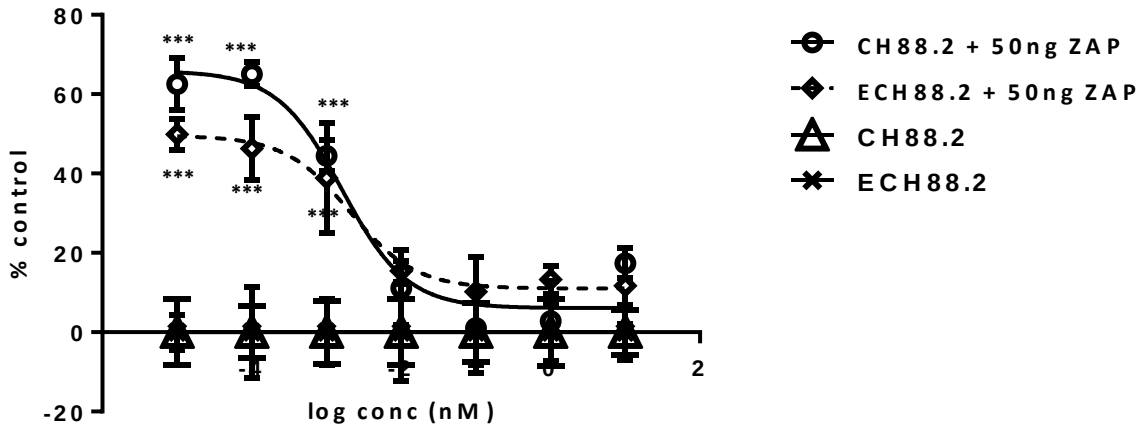


Figure 4.9: Targeted toxin (saporin) delivered by internalised CH88.2 and ECH88.2 mAbs in HCT15 cells. The anti-proliferative effect of internalised CH88.2 and ECH88.2 mAbs pre-incubated with a saporin-linked anti-human IgG Fab fragment was evaluated using 3H-thymidine incorporation. MAb concentration is shown on the x-axis. Results are presented as a percentage of the proliferation of cells treated with the primary mAbs only. Non-linear regression and significance between mAbs with

a range of concentration with untreated cells was established by two way ANOVA, GraphPad Prism 6.

4.3: Discussion

During the last two decades, mAb humanisation has solved a large number of problems associated with murine mAb such as immunogenicity, poor effector functions, and pharmacokinetics. In the previous chapter, the generation and characterisation of the murine IgG3 FG88.2 mAb was described. The FG88.2 mAb had high binding affinity and mediated excellent ADCC and CDC of tumour cells. Importantly, FG88.2 mAb also induced potent direct tumour cell death via an oncosis-like cell death. However, in this chapter, it is shown that the chimeric version of the mAb (CH88.2) had a reduced functional affinity and lost its direct killing ability. The low functional affinity of the IgG1 CH88.2 could be attributed to the loss of the ability to form non covalent interactions between mAbs. In mice and humans (Abdelmoula et al., 1989; Gyotoku, Abdelmoula, Spertini, Izui, & Lambert, 1987) (Panka, 1997), it is well known that IgG3 antibodies but not IgG1 and IgG2b antibodies, which express the same V domains (Greenspan & Cooper, 1993) can self-associate (cooperative binding) via Fc-Fc interactions (Abdelmoula et al., 1989; Gyotoku et al., 1987; Panka, 1997). Greenspan *et al.* described cooperative binding between IgG3 antibodies based on, for example, binding to highly repetitive antigens such as carbohydrates, the antibodies could have the same or different specificities but they must be of the mouse IgG3 isotype and the antigen must be densely packed (Greenspan & Cooper, 1993). Panka claimed that the CH3 domains of murine IgG3 antibodies were involved in this interaction (Panka, 1997). The presence of an additional glycosylation site at residues 471-473 in the murine IgG3 CH3 domain was found to be crucial for the self-association (Panka, 1997). The Fc-mediated self-association could be a mechanism by which functional affinity is increased, in particular to multivalent antigens to which antibodies with low intrinsic affinities are raised. Recently, Diebolder *et al.* demonstrated that introducing the E345R mutation conferred an enhanced ability to form hexamer structures via non covalent interactions involving the Fc region of mAbs. The engineered mAbs induced more potent CDC than the unmodified mAbs (Diebolder et al., 2014). In this study, it has been shown that the introduction of the E345R mutation increased functional affinity, CDC, ADCC and restored the direct killing ability of the CH88 mAb.

The ECH88.2 has shown enhanced functional affinity compared to CH88.2 mAb. This phenomenon could be attributed to multivalent binding of the antigen due to non-covalent interactions between ECH88.2 mAbs in the presence of its target, which enables ECH88.2 to compensate for intrinsic low monovalent affinity (Yoo, Wims, Chan, & Morrison, 2003). In addition to the increase in antibody avidity, ECH88.2 mAb also established the ability of the parental FG88.2 mAb to induce direct cell death of tumour cells. BR96 is an internalising murine IgG3 mAb recognising $Le^{y}Le^{b}$ glycoepitopes

(Noble, Spendlove, Harding, Parsons, & Durrant, 2013) overexpressed on colorectal, breast, lung and ovarian tumours (Hellstrom, Garrigues, Garrigues, & Hellstrom, 1990). Like FG88.2 mAb, murine BR96 has been shown to induce excellent immune mediated cytotoxicity as well as oncosis-like direct tumour cell death (Hellstrom et al., 1990). In contrast, the human IgG1 chimeric antibody BR96 (ChiBR96) lost its direct cytotoxic effect against tumour cells at 37°C (Wolff, Schreiber, Cosand, & Raff, 1993). Heterobifunctional cross-linkers were used to construct ChiBR96 homodimers which showed approximately 150 fold greater functional affinity compared to the monomers due to a slower antibody dissociation rate (Wolff et al., 1993). More importantly, the homodimers restored direct cytotoxicity as well as improved internalisation which translated into an effective anti-tumour effect *in vivo* (Wolff et al., 1993).

The most effective human antibody isotypes for stimulating the classic complement cascade were identified to be IgG3 followed by IgG1 (Strome et al., 2007). In this study, we have shown that the E345R mutation enhanced the CDC activity of ECH88.2 mAb compared to CH88.2 mAb. Diebolder *et al.* demonstrated that, by introducing the E345R mutation into human IgG mAb, specific non-covalent interactions between Fc segments of the engineered mAbs resulted in the formation of ordered antibody hexamers after antigen binding on cells. These hexamers recruited and activated C1q thereby triggering the complement cascade better than their unmodified counterparts (Diebolder et al., 2014). Accumulated evidence indicates that CDC activity can be enhanced by modifying the antibody Fc region via amino acid mutation (Idusogie et al., 2001), either in the Fc region or in the hinge region (Dall'Acqua, Cook, Damschroder, Woods, & Wu, 2006), to improve C1q binding. Partial or whole deletion of the hinge sequences in human IgG3 antibodies were also shown to improve CDC activity (Brekke et al., 1993; Michaelsen, Aase, Westby, & Sandlie, 1990). The four antibody isotypes differ in their potencies of CDC activity (IgG3 ≥ IgG1 > IgG2 = IgG4) (Bruggemann et al., 1987; Niwa et al., 2005; Schumaker, Calcott, Spiegelberg, & Muller-Eberhard, 1976). In recent years, structural shuffling between isotypes to generate chimeric isotypes with altered functions have been studied (e.g. IgG1/ IgG3, IgG1/ IgG4, IgG2/ IgG3 or IgG3/ IgG4 shuffling) (Canfield & Morrison, 1991; Natsume et al., 2008; Norderhaug et al., 1991; L. K. Tan, Shopes, Oi, & Morrison, 1990; Tao, Canfield, & Morrison, 1991; Zuckier, Chang, Scharff, & Morrison, 1998). However, these modifications rarely generated enhanced CDC activity compared to wild type antibody with the exception of the IgG1/IgG3 mixed isotype reported by Natsume et al, which was generated by replacing the CH1 domain of hIgG3 with the CH1 domain of hIgG1. This IgG1/IgG3 mixed isotype mAb was found to exhibit significant enhancement in CDC activity compared to parental antibodies both *in vitro* and *in vivo* (Natsume et al., 2008). Both CH88.2 and ECH88.2 mAbs also induced potent

ADCC. Interestingly, ECH88.2 showed stronger ADCC when compared to CH88.2 mAb, suggesting that the E345R mutation may also result in improved ADCC activity.

Two independent approaches demonstrated efficient internalisation of CH88.2 and ECH88.2 mAbs into HCT15 cells, namely confocal microscopy and cell killing with Fab-ZAP conjugates. This also indicates the potential of this mAb for ADC development. Conventional anti-cancer therapeutics often lack specificity, resulting in substantial toxicities to normal healthy tissues (Casi & Neri, 2012). MAbs display preferential binding to tumour cells, but, in isolation, their curative potential is often limited. Antibody drug conjugation is an approach which combines the tumour specificity of mAbs together with the potent cytotoxicity of cytotoxic drugs, aiming to reduce systemic toxicity but increase the therapeutic benefit for patients (Casi & Neri, 2012; Lambert & Chari, 2014).

Despite some promising results, the assumption that immune effector functions (ADCC and CDC) dominate the anti-tumour effects elicited by most therapeutic mAbs for tumour elimination may be in need of revision (Rutkowski, Sughrue, Kane, Mills, & Parsa, 2010). Accumulated evidence indicates that the tumour microenvironment can suppress the cytotoxic function of immune effector cells. Many factors have been proposed for the functional inactivation of tumour associated NK cells, which are the main mediator of ADCC (Han, Papadopoulos, Ruparelia, Devaja, & Raju, 1997; Miescher et al., 1988; Qin, Han, & Pang, 1997; Tsuboi, Tanaka, Nakao, Shichijo, & Itoh, 1995). Macrophages are another key player in eliminating tumours via ADCP (Josephs, Bax, & Karagiannis, 2015). Two distinct subsets of macrophages have been proposed, the classically activated type 1 (M1) and alternatively-activated type 2 (M2) phenotypes (Quatromoni & Eruslanov, 2012). In the immunosuppressive tumour microenvironment, macrophages have a predominantly M2-phenotype (tumour associated macrophages; TAMs) (Josephs et al., 2015) and clinical and experimental evidences have shown that the M2 macrophages are drivers of tumour progression (Hagemann, Balkwill, & Lawrence, 2007; Josephs et al., 2015; Mantovani & Allavena, 2015; Qian & Pollard, 2010). The efficacy of cancer immunotherapy with complement activating mAbs is limited by the overexpression of membrane complement regulatory proteins (mCRPs; CD46, CD55 and CD59) on the tumour cell surface(Campbell & Morgan, 1985; Fishelson et al., 2003; Morgan, 1989; Muller-Eberhard, 1988). Many groups have observed that the more mCRPs that are expressed on tumour cells, the more they are resistant to killing by locally activated complement (Weichenthal et al., 1999).

In summary, although therapeutic mAbs with a direct anti-tumour effect have shown promising result in eliminating tumours, due to the genetic variability typical of tumours and their immune evasion capacity of tumours, mAbs with multiple effector mechanisms may be essential to achieve

maximal anti-tumour effects. Previously, we have shown that the parental FG88.2 mAb induced potent *in vitro* cytotoxicity on tumour cells via immune effector functions (ADCC and CDC) and direct killing, which translated into significant survival improvement in a colorectal hepatic metastasis xenograft model. In this study, we demonstrate a novel result: endowing non-covalent interaction between ECH88.2 mAbs in the presence of antigens by introducing E345R mutation at Fc region of the mAb leads to increased binding to cell surface antigens and restores the direct killing ability of the mAb. Importantly, the mutation improves both the ADCC and the CDC activity of the mAb. Altogether these results suggest ECH88.2 mAb may have great potential for cancer therapy.

Chapter 5: Roles of FG88.2 glycoepitopes expressed on glycolipids and glycoproteins

5.1: Background

In the previous chapters, FG88.2 mAb was shown to bind to the novel Le^cLe^x, di-Le^a and Le^aLe^x glycoepitopes, which can be present on glycolipids or glycoproteins. FG88.2 induces potent direct cell death as well as internalising into high antigen expressing tumour cells. To dissect the relative role of glycolipids versus glycoproteins in FG88.2 mAb direct killing and internalisation, a study was conducted using LMTK cells, which only express lactosyl ceramide [(Cid, Yamamoto, Buschbeck, & Yamamoto, 2013); Figure 5.1], transduced with the glycosyltransferases necessary to express Le^a preferentially on glycolipids (G6B3R9) and on glycoproteins (G6B14R9).

The G6B3R9 cell line was produced by transducing wild type LMTK cells with the Lc3 synthase (B3gnt5), β-1,4-galactosyltransferase (β3Gal-T1) and α-1,4-fucosyltransferase (FUT3) genes (Figure 5.1). The G6B14R9 cell line was produced by transducing LMTK cells with the N-acetylgalactosaminyltransferase, β-1,3-acetylglucosaminyltransferase 6, β-1,4-galactosyltransferase (β3Gal-T1) and α-1,4-fucosyltransferase (FUT3) genes (Figure 5.1). Both cell lines were generous gifts from Dr Emili Cid and Dr Fumiichiro Yamamoto (IJC Josep Carreras Leukemia Research Institute, Barcelona).

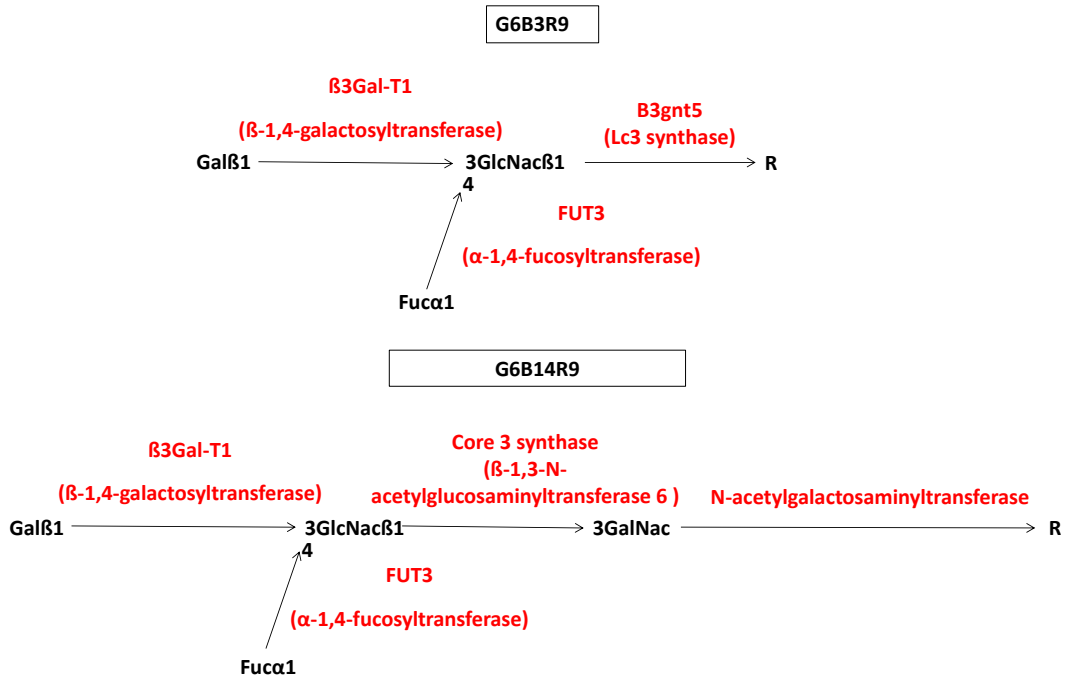


Figure 5.1: Biosynthetic pathways of the Lewis a carbohydrate antigen preferentially on glycolipids (G6B3R9) and on glycoproteins (G6B14R9). Glycan intermediates are shown in black font and enzymes responsible for sugar additions are shown in red font.

5.2: Results

5.2.1: Characterisation of the G6B3R9 and G6B14R9 cell lines

To determine whether the G6B3R9 and G6B14R9 cells expressed glycoepitopes recognised by FG88.2 mAb, the binding of FG88.2 mAb to these cell lines was determined by indirect immunofluorescence and flow cytometric analysis (Figure 5.2). HCT15 and LMTK cells were included as positive and negative control cell lines, respectively. FG88.2 mAb showed comparable binding level to G6B3R9, G6B14R9 and HCT15 cells (G6B3R9 Gm: 136; G6B14R9 Gm: 111; HCT15 Gm: 150) but did not bind to the LMTK wild type cells (Gm: 15). Medium alone (RPMI) was used as the negative control (G6B3R9 Gm: 6; G6B14R9 Gm: 10; HCT15 Gm: 10; LMTK Gm: 8).

FG88.2 mAb bound to glycoepitopes expressed on glycoproteins (MW ranging from 10 to > 230kDa) on Colo205, G6B14R9 and G6B3R9 but not on, AGS or LMTK cell lysates. It bound to glycolipids on G6B3R9 and Colo205 cells but not on G6B14R9, AGS or LMTK. FG88.2 mAb showed similar levels of glycolipid recognition but much lower levels of glycoprotein recognition on G6B3R9 cells compared to Colo205 cells. Importantly, FG88.2 mAb bound only glycoproteins on G6B14R9 cells. No antibody reactivity was seen with the AGS and LMTK negative control cell lines. No primary antibody was included as a negative control (Figure 5.3).

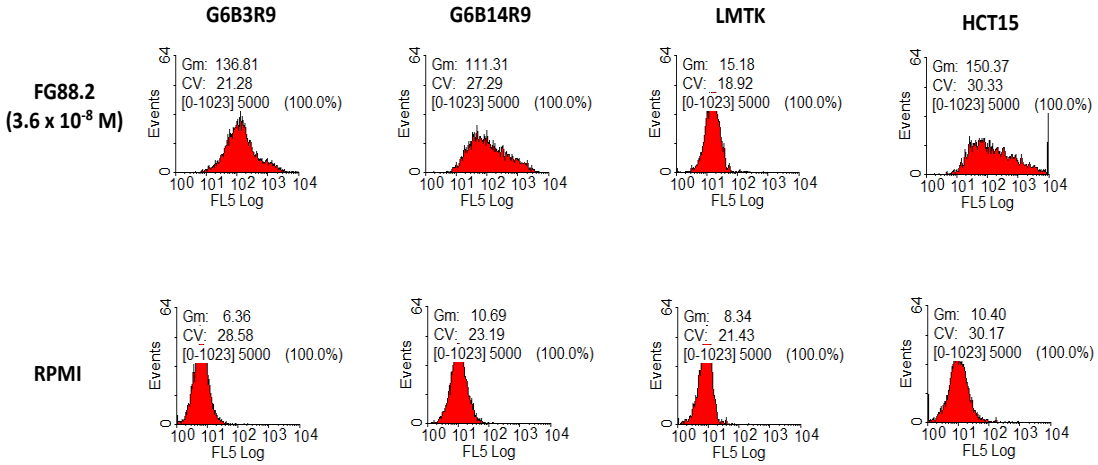


Figure 5.2: Binding of FG88.2 mAb to cell surface antigens. Binding of FG88.2 (3.6 x 10^{-8} M) to G6B3R9, G6B14R9, HCT15 and LMTK cells was assessed by indirect immunofluorescence staining and flow cytometric analysis. Results are expressed as geometric mean values (Gm).

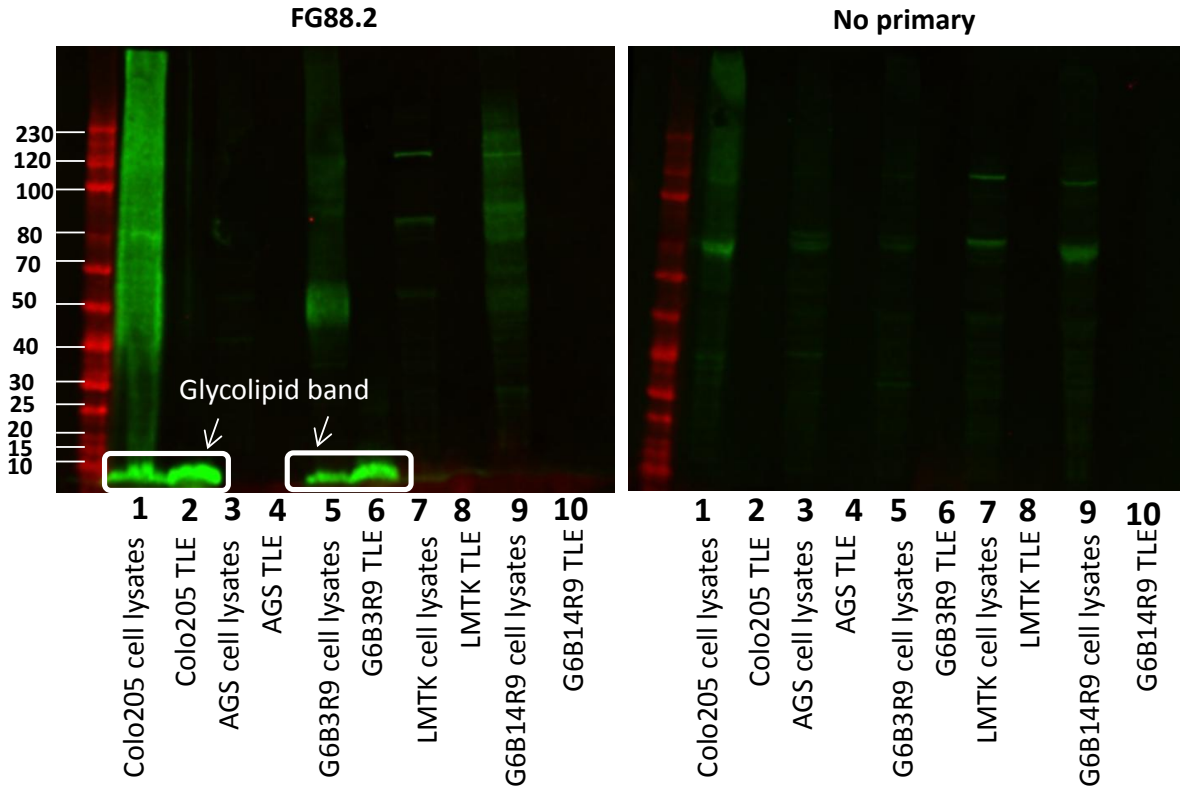


Figure 5.3: Binding of FG88.2 mAb to protein and lipid antigens was assessed by Western blot analysis. PAGE gel electrophoresis and Western blot analysis of cell lysates (5 x 10^5 cells equiv.) of 1) Colo205, 3) AGS, 5) G6B3R9, 7) LMTK and 9) G6B14R9 cells; and total lipid extracts (TLE) (1 x 10^6 cells equiv.) of 2) Colo205, 4) AGS, 6) G6B3R9, 8) LMTK and 10) G6B14R9 cells using FG88.2 mAb at 3.6 x 10^{-8} M. No primary antibody was included as a negative control.

5.2.2: The glycoepitopes expressed on glycolipids are responsible for the direct cytotoxicity activity of the FG88.2 mAb

The role of FG88.2 glycoepitopes expressed on glycolipids and glycoproteins in the direct killing ability of the mAb was investigated. FG88.2 mAb was tested for direct induction of PI uptake on G6B3R9 (Le^a preferentially on glycolipids) and G6B14R9 (Le^a on glycoproteins) cells at 37°C (Figure 5.4). For comparison, the HCT15 cell line that is susceptible to FG88.2 mAb direct killing was included. LMTK was used as a negative control cell line. Hydrogen peroxide (H_2O_2) and medium alone (RPMI) were included as positive and negative controls respectively. FG88.2 mAb induced 77% (2.2 x 10^{-7} M) and 40% (7.3 x 10^{-8} M) PI uptake in HCT15 cells, and 75% (2.2 x 10^{-7} M) and 51% (7.3 x 10^{-8} M) PI uptake in G6B3R9 Le^a glycolipid expressing cells. In contrast, FG88.2 mAb failed to induce PI uptake in G6B14R9 Le^a glycoprotein expressing cells. This suggests that the direct killing is predominantly mediated by glycolipids.

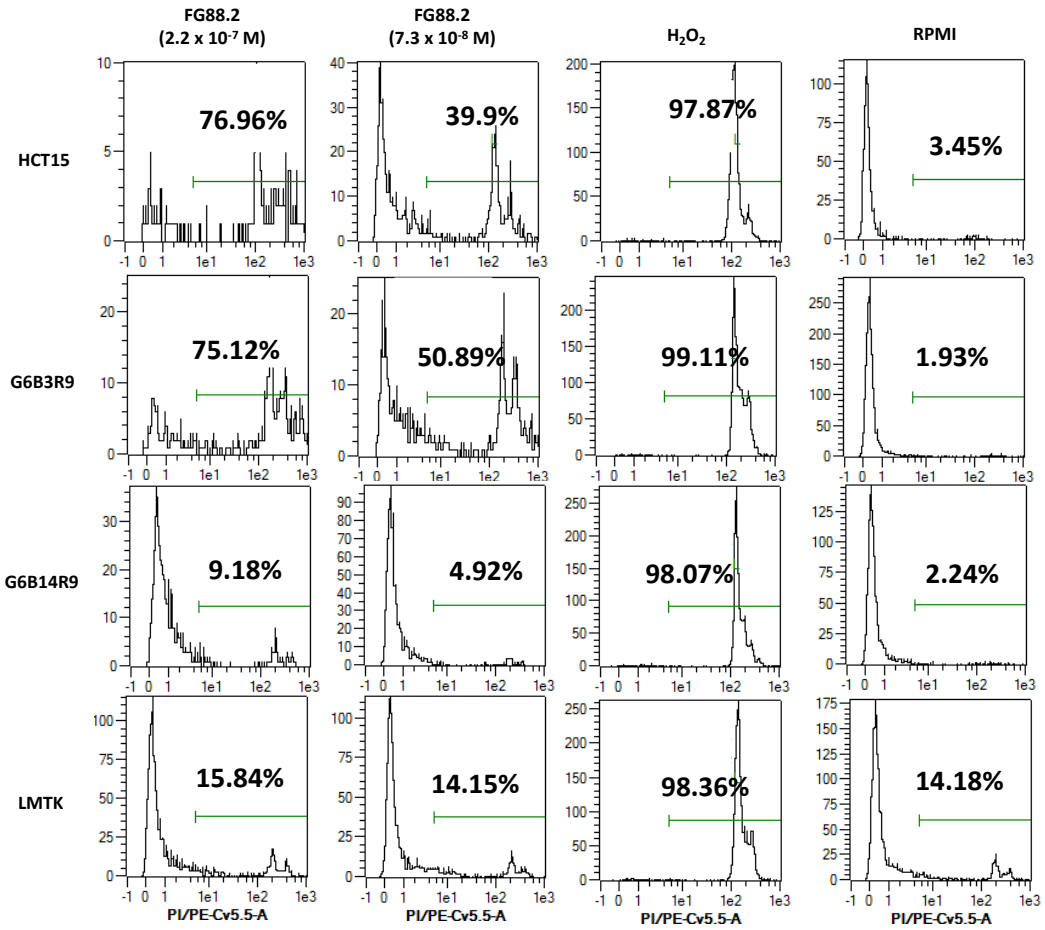


Figure 5.4: FG88.2 mAb induced PI uptake in HCT15 and G6B3R9 cells but not G6B14R9 and LMTK cells at 37^{0}C. HCT15, G6B3R9, G6B14R9 and LMTK cells were incubated with FG88.2 mAb at 2.2 x 10^{-7} M and 7.3 x 10^{-8} M at 37^{0}C and PI uptake determined by flow cytometry. Hydrogen peroxide (H_2O_2) and medium alone (RPMI) were included as positive and negative controls respectively.

To confirm that the PI assay truly reflects cell death, the cell viability of G6B3R9 (Le^a preferentially on glycolipids) and G6B14R9 (Le^a on glycoproteins) cells treated with FG88.2 was investigated using a WST8 proliferation assay (Figure 5.5). HCT15 cells were included as a positive control cell line. FG88.2 mAb-treated G6B3R9 (Le^a preferentially on glycolipids) and HCT15 cells revealed significant dose-dependent growth inhibition with an IC_{50} of 3.3 x 10^{-8} M and 6.2 x 10^{-8} M respectively. FG88.2 failed to show significant inhibition of G6B14R9 (Le^a on glycoproteins) cell growth again suggesting that growth inhibition was glycolipid mediated.

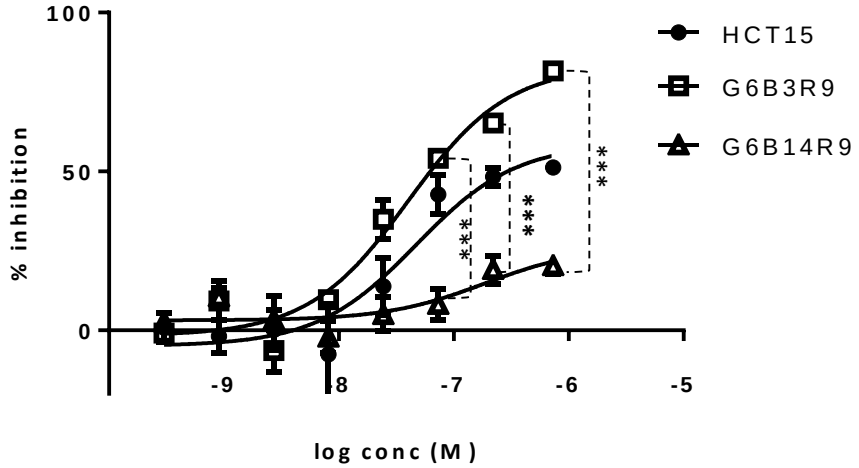


Figure 5.5: Growth inhibition effect of FG88.2 on G6B3R9 (Le^a preferentially on glycolipids) and G6B14R9 (Le^a on glycoproteins) cells. The anti-proliferative effect of FG88.2 mAb was analysed using the WST-8 assay. G6B3R9 and G6B14R9 cells were incubated with FG88.2 mAb for 96 hr after which WST-8 reagent was added following the manufacturer's instructions. The HCT15 cell line was included as positive control. Results are expressed as percentage inhibition compared to control cells. Statistical significance between data values from various cell lines was established by two way ANOVA, GraphPad Prism 6.

5.2.3: Both glycolipids and glycoproteins contribute to the FG88.2 mAb internalisation into tumour cells

In chapter 3, the cellular uptake and intracellular distribution of Alexa Fluor 488-labelled FG88.2 (A-FG88.2) mAb was studied using confocal microscopy. A-FG88.2 mAb was readily internalised into HCT15 cancer cells within minutes and co-localised with lysosomal compartments. In this study, we hypothesised that the binding of A-FG88.2 mAb to HCT15 cells increases the mobility of the plasma membrane, thus permitting the clustering of FG88.2-glycoepitopes. This phenomenon of FG88.2-glycoepitopes clustering was visualised via confocal microscopy over a 2 hr period (Figure 5.6). For FG88.2-treated HCT15 cells, A-FG88.2 mAb binding was initially distributed evenly throughout the cells, after 80 min the mAb was redistributed in small clusters and eventually large clusters on the HCT15 cell surface (Figure 5.6a). Interestingly, this perturbation of the cell membrane was followed by transient pore formation on HCT15 cells which resealed after A-FG88.2 mAb was internalised into the HCT15 cells (Figure 5.6b).

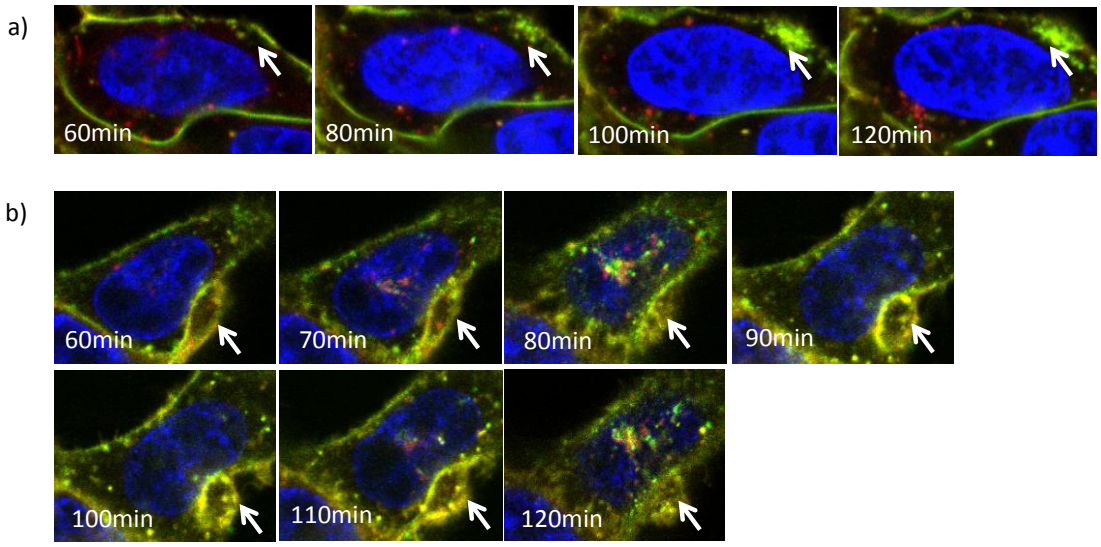


Figure 5.6: Time-lapse confocal microscopy of Alexa Fluor 488-labelled FG88.2 mAb internalising into live HCT15 cells. Merged images taken every 10 to 20 min demonstrate a) clustering of FG88.2-glycoepitopes on the HCT15 cell membrane and b) transient pore formation in the HCT15 cell membrane prior to A-FG88.2 mAb internalisation. The nucleus was labelled with Hoechst 33258, lysosomal compartments with LysoTracker Deep Red and the plasma membrane with CellMask Orange (*magnification x60*).

Subsequently, the role of FG88.2 glycoepitopes on glycolipids and glycoproteins on FG88.2 mAb internalisation was determined using the G6B3R9 (Le^a preferentially on glycolipids) and G6B14R9 (Le^a on glycoproteins) cell lines. The LMTK cell line was included as negative control cell line. Internalisation was validated through toxicity of Fab-ZAP-FG88.2 immune complex containing saporin (Figure 5.7). Internalisation of the Fab-ZAP-FG88.2 complex, but not the FG88.2 mAb alone, led to a dose dependent decrease in cell viability of both G6B3R9 (Ic_{50} 10^{-11}M) and G6B14R9 (Ic_{50} 10^{-10}M) cells suggesting that FG88.2-glycoepitopes on either/or glycolipids and glycoproteins are involved in FG88.2 mAb internalisation.

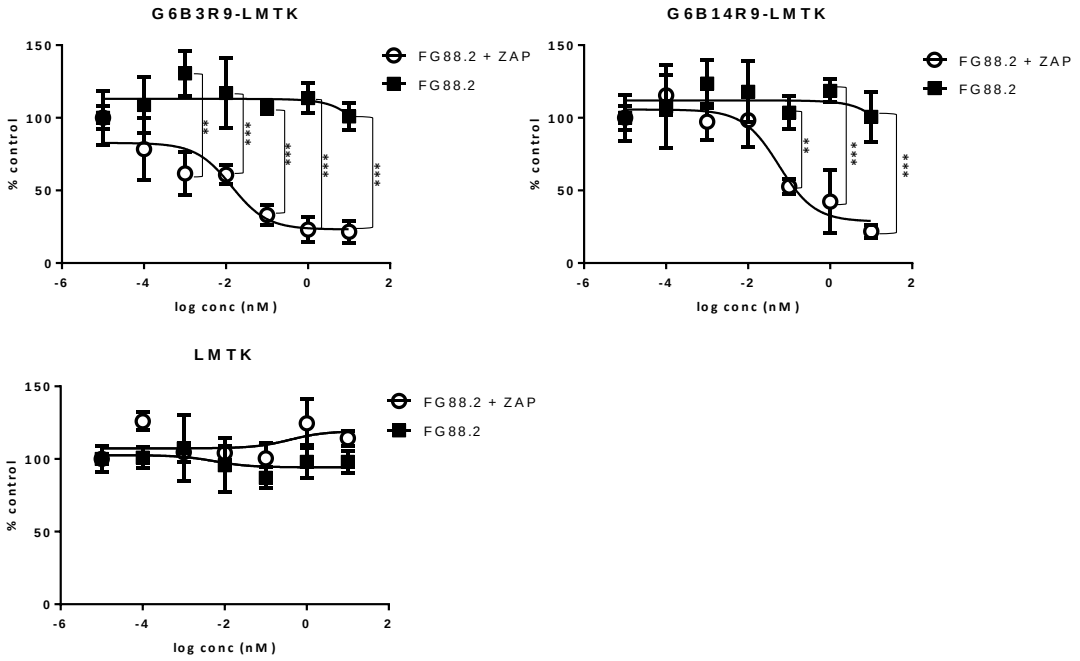


Figure 5.7: Targeted toxin (saporin) delivered by internalised FG88.2 mAb in G6B3R9 and G6B14R9 cells. The anti-proliferative effect of internalised FG88.2 mAb pre-incubated with a saporin-linked anti-mouse IgG Fab fragment was evaluated using ^{3}H-thymidine incorporation. Concentrations refer to FG88.2 mAb. Results are presented as a percentage of the proliferation of cells treated with primary mAb only. Non-linear regression and significance between mAbs with a range of concentrations was established by two way ANOVA, GraphPad Prism 6. Results are representative of three independent experiments.

5.3: Discussion

It was previously shown that the FG88.2 mAb was able to kill and internalise into tumour cells that expressed a high level of FG88.2-glycoepitopes. These FG88.2 glycoepitopes are co-expressed on both glycolipids and glycoproteins overexpressed on tumour cells. In this study, the contribution of glycolipids and/or glycoproteins towards the direct killing and internalising ability of FG88.2 mAb was investigated on the G6B3R9 (Le^{a} preferentially on glycolipids) and G6B14R9 (Le^{a} on glycoproteins) cell lines.

In this study, although FG88.2 mAb showed similar cell surface antigen binding to HCT15, G6B3R9 and G6B14R9 cells, only HCT15 and G6B3R9 (Le^{a} preferentially on glycolipids) cells were susceptible to FG88.2 mAb direct killing but not G6B14R9 (Le^{a} on glycoproteins) cells. This result ruled out the possibility that G6B14R9 cells expressing only low to moderate level of FG88.2-glycoepitopes and suggesting that FG88.2 glycoepitopes on glycolipids are key players in inducing direct tumour cell death.

FG88.2 mAb was shown to induce tumour direct cell death. Oncosis is a progressive cell death process initially involving the impairment of ionic pumps of the cell membrane accompanied by cellular and organelle swelling. Subsequently, a gradually increase in membrane permeability due to an increasing cytosolic calcium concentration as well as rearrangement of cytoskeletal proteins, results in pore formation in the cell membrane (Weerasinghe & Buja, 2012; Weerasinghe, Hallock, Brown, Loose, & Buja, 2013). In a study analogous to the FG88.2 mAb results described here, Tan *et al.* demonstrated that, mAb 84, when bound to human embryonic stem cells, induced cytoskeletal protein (α-actinin, paxillin and talin) degradation, which in turned increased the mobility of the plasma membrane, resulting in the clustering of antigens on the cell surface. Following antigen clustering, formation of pores through the plasma membrane resulted in oncosis (H. L. Tan et al., 2009).

Confocal microscopy of FG88.2-treated HCT15 cells showed clusters and pore formation in cell membranes followed by mAb internalisation and lysosomal localisation of a proportion of the FG88.2 mAb. FG88.2 mAb is a murine IgG3 mAb. The murine IgG3 isotype has been shown to form non covalent interaction between mAbs. Therefore, the clusters observed on FG88.2-treated cells could possibly be due to the surface antigen cross-linking by FG88.2 mAb. In eukaryotic cells, gangliosides (Bektas & Spiegel, 2004; Heffer-Lauc, Lauc, Nimrichter, Fromholt, & Schnaar, 2005) and GPI-anchored proteins (Harder, Scheiffele, Verkade, & Simons, 1998; Mayor, Rothberg, & Maxfield, 1994) have been reported to distribute asymmetrically in the cell membrane until they are cross-linked by viruses or antibodies. They then form clusters with cholesterol producing lipid microdomains (R. G. Anderson & Jacobson, 2002). In the cell membrane, lipid microdomains have a function in both endocytosis and cell signalling (R. G. Anderson & Jacobson, 2002). Examples of lipid microdomains are lipid rafts and caveolae. Lipid rafts are a collection of membranes that are rich in cholesterol, sphingomyelin and glycolipids. Due to their high lipid content, lipid rafts are insoluble in non-ionic detergents at 4°C; as well as having light buoyant density on sucrose gradients (R. G. Anderson & Jacobson, 2002; Simons & Ikonen, 1997). One group of membranes that have light buoyant density on sucrose gradients are caveolae (Sargiacomo, Sudol, Tang, & Lisanti, 1993). Caveolae are plasma membrane invaginations (Parton & del Pozo, 2013) that are characterised by a 'cup-shaped,' highly uniform structure (Richter et al., 2008). Caveolae are known to be enriched in specific sphingolipids (sphingomyelin, GD3, GM1 and GM3)(Ortegren et al., 2004; Pitto et al., 2000), cholesterol (Fujimoto, Hayashi, Iwamoto, & Ohno-Iwashita, 1997) and phosphatidylinositol (Fujita, Cheng, Tauchi-Sato, Takenawa, & Fujimoto, 2009).

The Fab-ZAP-FG88.2 internalisation assay showed efficient FG88.2 internalisation into both G6B3R9 and G6B14R9 cells suggesting both Le^a-glycolipids and Le^a-glycoproteins maybe involved in antibody-antigen complex internalisation. At this stage, the internalisation pathway that the FG88.2 mAb-antigen complex uses to enter cells remains unexplored. Endocytosis is a fundamental process in eukaryotic cells by which cells internalise membrane proteins, lipids, extracellular ligands and soluble molecules. The most well characterised internalisation pathways are clathrin-dependent (receptor-mediated endocytosis) and clathrin-independent endocytosis (phagocytosis, micropinocytosis and caveolin-dependent endocytosis) pathways (Ritchie, Tchistiakova, & Scott, 2013). Clathrin-dependent endocytosis involves the recruitment of AP2 adaptor protein, which recognises the cytoplasmic domains of plasma membrane receptors. They are packaged into clathrin-coated vesicles that are endocytosed into cells. Clathrin-dependent endocytosis is regulated by a range of accessory proteins (epsin, SNX9, synaptojanin, actin amphiphysin) and requires the GTPase dynamin for vesicle scission (Doherty & McMahon, 2009; B. D. Grant & Donaldson, 2009). In contrast to clathrin-dependent endocytosis, clathrin-independent endocytosis does not appear to require the formation of the clathrin coat. The most commonly reported clathrin-independent endocytosis has been caveolae (Doherty & McMahon, 2009). Caveolins, which are a family of integral membrane proteins work together with cavin family proteins (Cavin1-4), to form the principle membrane components of caveolae ((Bastiani & Parton, 2010; Parton & del Pozo, 2013). Caveolae have close association with actin cytoskeleton via the interaction between actin-binding protein filamins with cavin proteins (Stahlhut & van Deurs, 2000). Regardless of the mode of entry, endocytosed cargo is usually delivered to the early endosome where sorting occurs. The cargo can be sent to late endosomes and eventually lysosomes for degradation, to the trans-Golgi network, or to recycling endosomal carriers that recycles cargo back to the cell membrane (B. D. Grant & Donaldson, 2009).

Several studies have demonstrated that GSLs are able to enter cells via both clathrin-dependent and clathrin-independent pathways. For example, cholera toxin-GM1 complex has been shown to enter cells via both pathways, depending upon caveolin-1 expression levels in different cell types (Shogomori & Futerman, 2001; Singh et al., 2003; Torgersen, Skretting, van Deurs, & Sandvig, 2001; Tran, Carpentier, Sawano, Gorden, & Orci, 1987). Cholera toxin-GM1 complex internalises via clathrin-independent endocytosis in cells with high caveolin-1 expression level; whereas significant clathrin-dependent endocytosis is observed in cells with low caveolin-1 expression (Singh et al., 2003). In contrast to GSLs, it was believed that clathrin-dependent endocytosis pathway was the major pathway for internalisation of most receptor tyrosine kinases such as EGFR, neurotrophic tyrosine kinase receptor type 1 (TrkA), insulin receptor (IR) (Beattie, Howe, Wilde, Brodsky, &

Mobley, 2000; Bogdanovic, Coombs, & Dumont, 2009; Carpentier et al., 1982; Gorden, Carpentier, Cohen, & Orci, 1978). Of all these receptors, EGFR appears to be the most studied receptor tyrosine kinase. EGFR has been reported to internalise efficiently via clathrin dependent pathway only in response to ligand-induced activation of its intrinsic tyrosine (Salazar & Gonzalez, 2002). Several groups have demonstrated that EGF-stimulated receptor endocytosis can be completely abolished with clathrin knockdown models (Grandal et al., 2012; Henriksen, Grandal, Knudsen, van Deurs, & Grovdal, 2013; Kazazic et al., 2006). In addition, Kazazic et al demonstrated that caveolae are not involved in EGF-EGFR complex internalisation and high concentration of EGF does not further mobilise caveolae (Kazazic et al., 2006).

Studies of the role of glycolipids and glycoproteins in inducing direct tumour cell death and mAb internalisation have highlighted important questions for further study. The downstream signalling pathway of oncolytic cell death and its ability to induce immunogenic cell death and induction of anti-tumour immunity is an area for future studies. Equally the mechanisms for recruitment of FG88.2 glycoepitopes on both glycolipids and glycoproteins at the cell membrane that lead to internalisation via clathrin dependent or independent endocytosis require further investigation. In addition, it is important to note that, although FG88.2 mAb internalises into tumour cells, the mAb was still able to kill tumour cells via ADCC and CDC, suggesting that in early endosomes, part of the internalised FG88.2 mAb was recycled back to the cell surface or that some mAbs remained on cell surface. Further research should be aimed towards the exploration of both oncosis and internalisation mechanisms induced by FG88.2 mAb.

Chapter 6: FG2811.72, an ultra-specific mouse monoclonal antibody against stem cell embryonic antigen-4 (SSEA-4).

6.1 Background

Glycosphingolipids (GSLs) are essential biomolecules of cell membranes and are composed of at least one monosaccharide and either a sphingoid or ceramide (Chester, 1998). GSLs are generally classified as neutral, sialylated, or sulphated based on differences in glycan component (Schnaar, 2004). Specifically, the major structural categories of vertebrate GSLs are: (i) the lacto- and neolacto; (ii) the ganglio- and isoganglio-; and (iii) the globo- and isoglobo-series (Liang et al., 2010). In addition to their role as structural components in the cell membrane, GSLs play crucial roles in biological functions such as cell adhesion, signal transduction, apoptosis, growth and differentiation (S. I. Hakomori, 2008; Z. Wang et al., 2012).

The aberrant glycosylation associated with tumour progression was first described in the 1960's with the demonstration that numerous glycans on malignant cells differ from normal cells (Meezan, Wu, Black, & Robbins, 1969). These changes include loss or overexpression of certain glycan epitopes, persistence of truncated glycans, and emergence of novel glycans (Huang et al., 2013). Many studies indicate that aberrant glycosylation is responsible for initial oncogenic transformation, as well as playing a key role in induction of tumour invasion and metastasis (S. Hakomori, 2002). To date, overexpression of a broad range of GSLs has been identified in various types of human malignancies: GD3 in melanoma (Nudelman et al., 1982), GD2 in neuroectodermal tumours (Cahan, Irie, Singh, Cassidenti, & Paulson, 1982), fucosyl-GM1 in small cell lung carcinomas (Nilsson et al., 1986), Globo-H in breast and ovarian carcinomas (Chang et al., 2008) and, SSEA-3 and SSEA-4 in breast cancer and breast cancer stem cells (Chang et al., 2008).

Stage specific embryonic antigens (SSEAs) are globoseries glycolipids and are composed of 3 species: SSEA-1, SSEA-3 and SSEA-4 (Suzuki et al., 2013). SSEA-1 and SSEA-4 were identified via immunisation of animals with human embryonic carcinoma cells (human teratocarcinoma cells; tumours containing tissue derivatives of all three germ-layers) whereas SSEA-3 was identified by immunisation with 4- to 8-cell stage mouse embryos (Kannagi et al., 1983; Shevinsky, Knowles, Damjanov, & Solter, 1982; Solter & Knowles, 1978; A. J. Wright & Andrews, 2009). SSEA-1, also known as Lewis x (Le^x) or CD15, is a trisaccharide (Galβ1→4(Fucα1→3)GlcNAc), which can be found on human polymorphonuclear granulocytes and various carcinomas such as colon and breast tumours (Elola et al., 2007). SSEA-3, also known as galactosyl globoside or Gb5Cer, is a pentasaccharide (Galβ1→3GalNacβ1→3Galα1→3Galβ1→4Glcβ1) (Andrews, 2011), and SSEA-4, also known as sialyl galactosyl globoside or sialyl Gb5Cer (NeuNACα2→

Galβ1→3GalNacβ1→3Galα1→3Galβ1→4Glcβ1) is similar to SSEA-3 in structure except that it has an additional terminal sialic acid moiety (Suzuki et al., 2013). SSEA-3 and SSEA-4 are widely used as cell surface markers to define human embryonic stem cells (hESCs) (Lou et al., 2014).

To our knowledge, MC631 (rat IgM anti-SSEA-3 mAb) and MC813-70 (mouse IgG3 anti-SSEA-4 mAb), are the only anti-SSEA-3 and anti-SSEA-4 mAbs commercially available. The potential cytotoxicity of MC631 mAb has not been reported. MC813-70 mAb has been shown to induce CDC against SSEA-4 high expression glioblastoma multiforme (GBM) cell lines *in vitro* and suppressed tumour growth *in vivo* (Lou et al., 2014). In the present study, we describe the generation and characterisation of a high affinity, internalising anti-SSEA4 mAb (FG2811.72 mAb), which displays effective immune-mediated cytotoxicity to breast and ovarian cancer cells. In addition, FG2811.72 mAb showed great potential to inhibit tumour cell growth in the absence of immune effector cells. We investigated FG2811.72 expression in breast and ovarian cancers to determine FG2811.72 mAb therapeutic potential in breast and ovarian cancers.

6.2: Results

6.2.1: Generation of murine FG2811.72 mAb

Three BALB/c mice (1R, 1P and 1L) were immunised intraperitoneally (i.p.) and boosted intravenously (iv.) over a period of 3 months with an SSEA-3 expressing cell line (SSEA-3-LMTK); produced by transducing wild type LMTK mouse fibroblast cells with α-1-4-galactosyltransferase (A4GALT), β-1-3-N-acetylgalactosaminyltransferase (B3GALNT1) and β-1-3-galactosyltransferase (B3GALT5) genes (Figure 6.1) (Cid et al., 2013). The immunisation protocol is shown in Table 6.1.

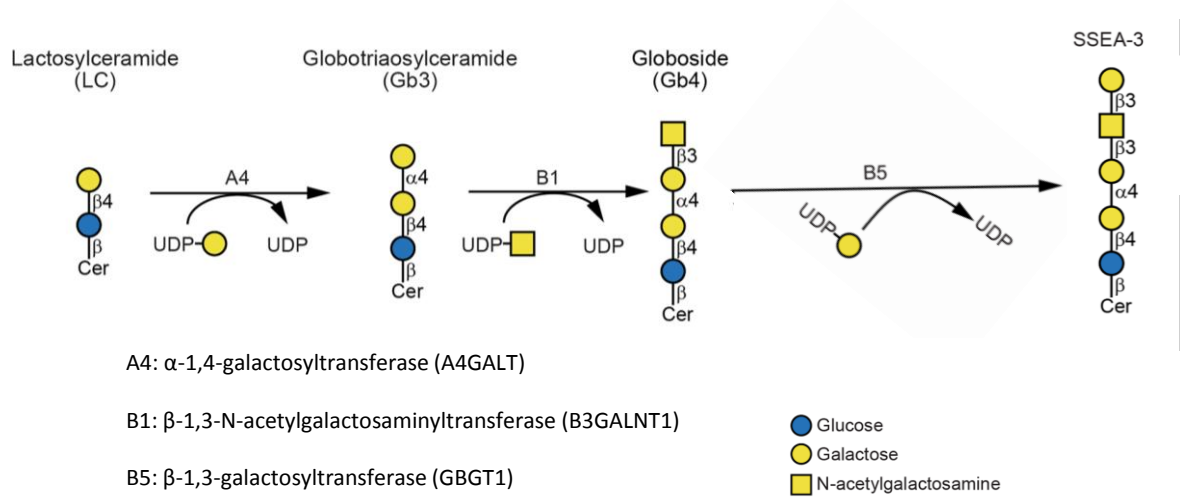


Figure 6.1: Schematic diagram of the generation of SSEA-3 glycans from lactosylceramide. (UDP: uridine diphosphate; Cer: ceramide) (Cid et al., 2013)

	Mouse 1R	Mouse 1L	Mouse 1P
1st immunisation	SSEA-3-LMTK cells (10^6 cells)	SSEA-3-LMTK cells (10^6 cells)	SSEA-3-LMTK cells (10^6 cells)
2nd immunisation	SSEA-3-LMTK cells (10^6 cells)	SSEA-3-LMTK cells (10^6 cells)	SSEA-3-LMTK cells (10^6 cells)
3rd immunisation	SSEA-3-LMTK cells (10^6 cells)	SSEA-3-LMTK cells (10^6 cells)	SSEA-3-LMTK cells (10^6 cells)
4th immunisation	SSEA-3-LMTK cells (10^6 cells)	SSEA-3-LMTK cells (10^6 cells)	SSEA-3-LMTK cells (10^6 cells)
Boost	SSEA-3-LMTK cells (10^5 cells)	-	-
Fusion	FG2811.72		

Table 6.1: Immunisation schedule.

To assess whether the immunised mice (1R, 1L and 1P) generated anti-glycolipid antibody responses, after the 2nd, 3rd and 4th immunisations, mice were tail bled and anti-sera were screened by ELISA against SSEA-3-LMTK lipid extracts. After four immunisations, all three mice demonstrated high IgM SSEA-3 lipid-antibody binding (end point titre: 1/10,000) (Figure 6.2). In contrast, only mouse 1R showed a weak IgG response to SSEA-3 lipid at 1/100 dilution (0.11 OD units) (Figure 6.2). Mice 1L and 1P did not show any specific IgG response. A biotinylated anti-SSEA-3 commercial mAb (MC631; rat IgM) was included as positive control (0.67 OD units). Normal mouse serum (NMS; untreated) (IgM; 0.12 OD units, IgG; 0.07 OD units) and PBS (0.02 OD units) were used as negative controls.

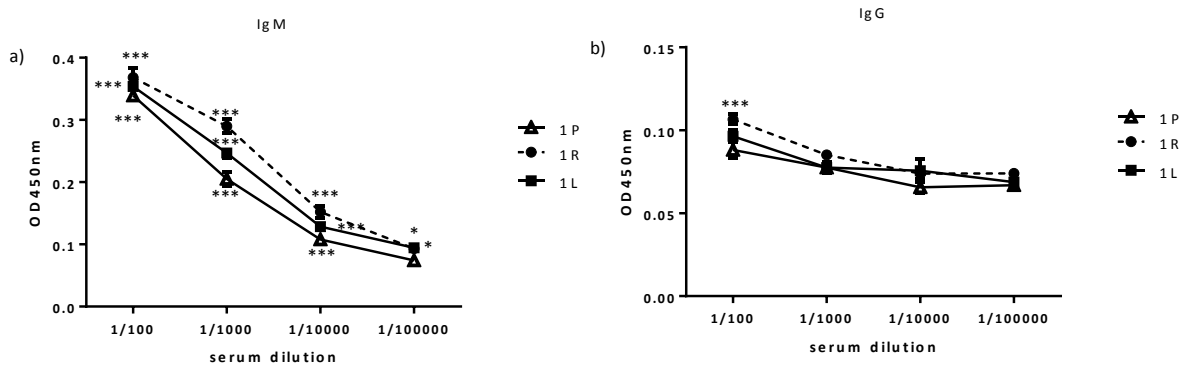


Figure 6.2: ELISA titration of immunised mice (1R, 1L and 1P) against SSEA-3-LMTK total lipid extract. SSEA-3-LMTK total lipid extract was dried onto a 96 well plate. Plates were blocked with PBS 2% (w/v) BSA before incubating with serial dilutions of mice sera (after 4 immunisations) and probed with biotinylated anti-mouse IgM or IgG Fc specific secondary antibody followed by detection with streptavidin-HRP. An anti-SSEA-3 commercial mAb (MC631) was included as a positive control whereas normal mouse serum (NMS) and PBS were used as negative controls. Error bars represent standard deviation of quadruplicate wells. Significance between mouse anti-sera and PBS control was established by one way ANOVA, GraphPad Prism 6 (*, P< 0.05; ***, P< 0.001 versus control).

Western blot analyses of SSEA-3-LMTK cell lysates and total lipid extract using the mouse anti-sera (1R, 1L and 1P) at 1/500 dilution demonstrated that IgG in all three mice recognised only a band at the dye front, which is consistent with a glycolipid (Figure 6.3). However, mouse 1L showed only a weak response. The low, intermediate and high molecular weight protein bands seen with the three mouse anti-sera were presumed to be background as normal mouse serum (NMS) showed similar protein recognition. No glycolipid reactivity was seen with NMS. The positive control mAb, MC631, also recognised the dye front band. Binding of all three mice anti-sera to SSEA-3-LMTK cells was shown by indirect immunofluorescence and flow cytometry analysis (Figure 6.4). All three mice anti-sera showed strong IgG and IgM antibody responses to SSEA-3-LMTK cell surface antigens at 1/100 dilution with 1P as the strongest binder for IgG response followed by 1R and 1L; (1P Gm: 301; 1R Gm: 277 and 1L Gm: 169). The IgG antibody-binding titre end point for all three mice sera was 1/1,000 dilution. In contrast, 1R was the strongest binder for IgM response followed by 1L and 1P; (1R Gm: 652; 1L Gm: 497 and 1P Gm: 397). In addition, 1R and 1L demonstrated the highest IgM antibody-binding titre (titre end point: 1/100,000) compared to 1P (1/1,000). MC631 mAb (IgM; Gm: 302) was used as positive control whereas NMS (1/100; Gm: 14), no primary (Gm: 13) and medium (RPMI; Gm: 12) were negative controls.

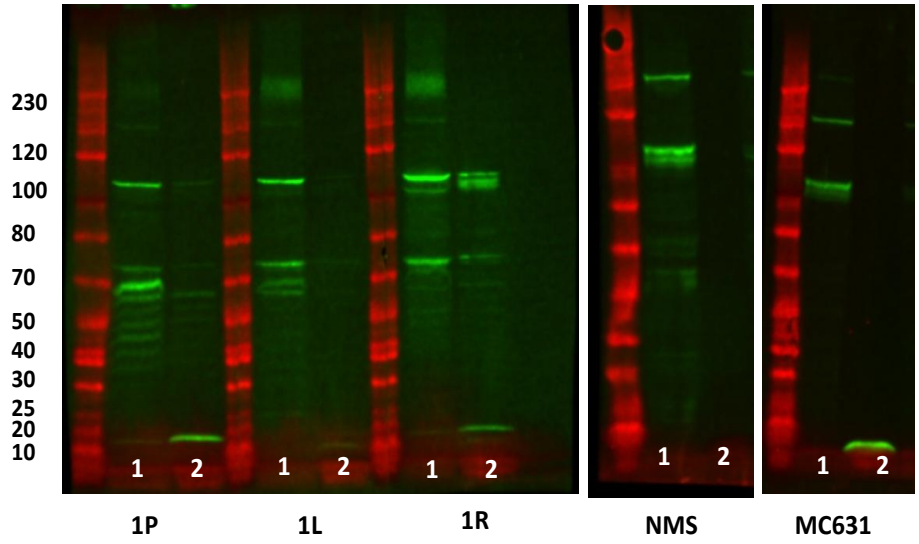


Figure 6.3: Binding of immunised mouse anti-sera (1L, 1R and 1P) to protein and lipid antigens were assessed by SDS-PAGE and Western blot analysis. SDS-PAGE and Western blot analysis of 1) SSEA3-LMTK cell lysates (2 x 10^5 cells equiv.) and 2) SSEA-3-LMTK total lipid extract (1 x 10^6 cells equiv.) using immunised mouse anti-sera (1L, 1R and 1P) at a 1/500 dilution. MC631 mAb (8 x 10^{-9} M) and NMS (1/500) were included as positive and negative control respectively (IgG response).

Mouse 1R was selected for the generation of hybridoma due to the IgG response in the SSEA-3 glycolipid ELISA, glycolipid recognition in Western blot analysis and the good IgG antibody titre in binding to SSEA-3-LMTK cell surface antigens. Mouse 1R was boosted with 10^5 SSEA-3-LMTK cells, sacrificed and the FG2811.72 mAb was generated by fusing splenocytes from mouse 1R with the mouse myeloma, NS0.

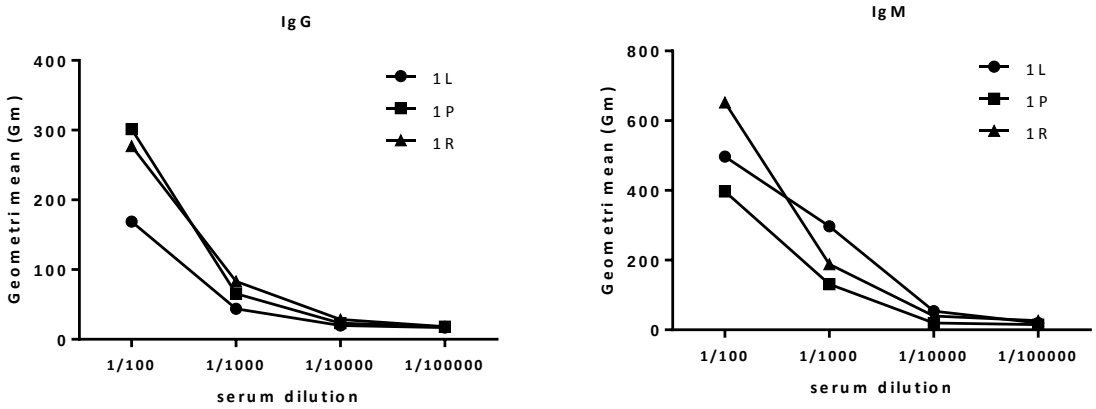


Figure6.4: Binding of mouse anti-sera (1L, 1P and 1R) to SSEA-3-LMTK cell surface antigens. Binding of mouse anti-sera to SSEA-3-LMTK cells were assessed by indirect immunofluorescence staining and flow cytometry analysis. In these assays, MC631 mAb (3.6 x 10^{-8} M) was included as a positive control whereas medium alone and NMS were included as negative controls.

6.2.2: Characterisation of FG2811.72 mAb

FG2811.72 is a mouse mAb with an IgG3 isotype, and κ light chains. FG2811.72 (hybridoma supernatant) and MC631 mAb (positive control) recognised glycolipids from SSEA-3-LMTK but not LMTK wild type cells by Western blot analysis (Figure 6.5). Three glycoprotein bands were confirmed as background bands compared to negative control (normal mouse serum; NMS; 1/100 dilution).

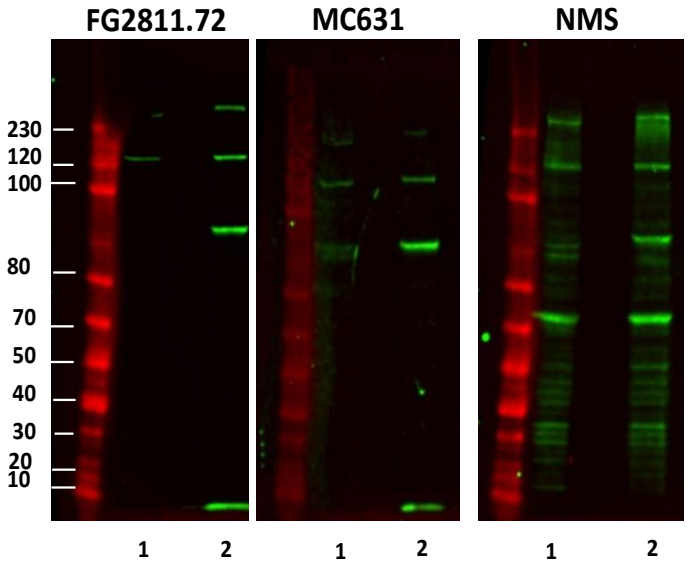


Figure 6.5: Binding of FG2811.72 mAb to protein and lipid antigens were assessed by PAGE and Western blot analysis. Western blot analysis of 1) LMTK cell lysates ($2x10^5$ cells equiv.) and 2) SSEA-3-LMTK cell lysates ($2x10^5$ cells equiv.) using FG2811.72 mAb (neat hybridoma supernatant). MC631 mAb (3.6×10^{-8} M) and normal mouse serum (NMS; 1/100 dilution) were used as positive and negative controls respectively.

FG2811.72 showed strong SSEA-3-LMTK cell-surface reactivity by flow cytometry analysis at 2.4×10^{-8} M (Gm: 3827) when compared to the positive control mAb, MC631 (2.4×10^{-8} M; Gm: 1294) and negative controls [no primary; Gm: 31 and medium alone (RPMI); Gm: 7] (Figure 6.6).

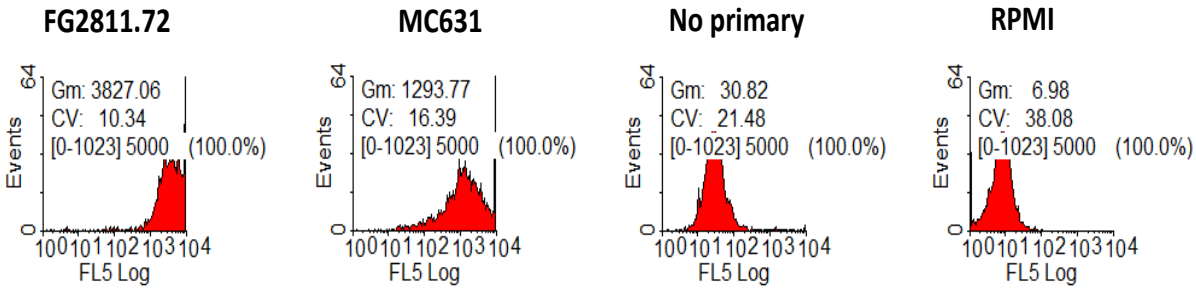


Figure 6.6: Binding of FG2811.72 mAb to SSEA-3-LMTK cell surface antigens. Binding of FG2811.72 (2.4×10^{-8} M) to SSEA-3-LMTK cells were assessed by indirect immunofluorescence staining and flow

cytometry analysis. Binding of FG2811.72 mAb was compared to the positive control, MC631 mAb (2.4 x 10^{-8} M); and the negative controls; no primary and medium alone (RPMI). Results are expressed as geometric mean values (Gm).

Confirmation of glycolipid reactivity came from TLC analysis of SSEA-3-LMTK plasma membrane lipid extracts and immunostaining with FG2811.72 mAb. MC631 mAb was included for comparison (Figure 6.7). As shown in Figure 6.7, both FG2811.72 and MC631 mAbs stained glycolipids expressed on SSEA-3-LMTK but not LMTK wild type cells, suggesting both mAbs stained SSEA-3 glycolipids expressed on SSEA-3-LMTK cells, not to any other antigens expressed on wild type LMTK cells. Importantly, FG2811.72 mAb showed very specific glycolipid staining whereas MC631 mAb stained three different glycolipid antigens suggesting the positive control mAb may cross-react with other glycolipid antigens. The result of MC631 mAb indicates that the SSEA-3-LMTK cells express not only SSEA-3 glycoepitope but possibly other related glycolipids.

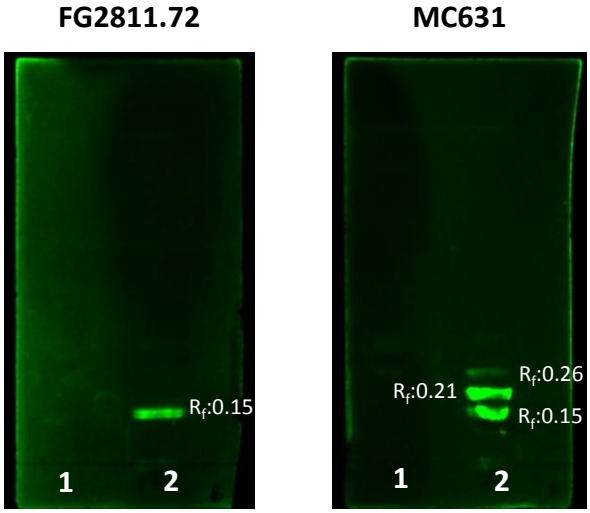


Figure 6.7: Binding of FG2811.72 mAb to lipid antigens was assessed by thin layer chromatography analysis. Thin layer chromatography analysis of 1) LMTK and 2) SSEA-3-LMTK plasma membrane lipid extracts (7.5x10^{6} cells equiv.) using FG2811.72 mAb (3.6 x 10^{-8} M) and MC631 mAb (1.8 x 10^{-8} M).

To identify the glycoepitopes recognised by FG2811.72 and MC631 mAbs, they were screened by ELISA against commercially obtained BSA-coupled SSEA-3 as well as SSEA-4, Forssman and Globo-H glycoepitopes (Figure 6.8 A and B). These are all similar to SSEA-3 in structure, except that SSEA-4 has an additional terminal sialic acid moiety; Forssman has an N-acetylgalactosamine (GalNAc) as the terminal glycan moiety rather than galactose; and Globo-H has an additional terminal fucose moiety. MC631 mAb was included as comparison. Secondary antibody alone (biotinylated-anti-mouse IgG antibody; negative control) did not show any reactivity with any of the glycans tested. Surprisingly, although FG2811.72 mAb was raised against SSEA-3-expressing cells, ELISA results showed that FG2811.72 mAb recognised SSEA-4 glycan strongly (1.2 OD units) and did not cross react with any

other glycans. MC631 mAb was commercialised as anti-SSEA-3 mAb, but in addition to SSEA-3 (1.0 OD units), it also recognised SSEA-4 strongly (0.97 OD units) and Globo-H moderately (0.53 OD units). MC813-70 mAb which was commercialised as anti-SSEA-4 mAb, recognised SSEA-4 strongly (1.18 OD units) but also strongly cross reacted with SSEA-3 (1.0 OD units) and Forssman (0.71 OD units); and weakly with Globo-H (0.12 OD units). M1/87 was used as positive control for Forssman (1.24 OD units) glycan and had the correct specificity. PBS alone was used as the negative control.

To clarify their fine specificities, FG2811.72 and MC631 mAbs (3.6×10^{-7} M) were screened against the glycan array from the Z Biotech, LCC, which is composed of 15 glycan targets, well characterised by glycan-binding proteins (lectins) (Table 6.2). Binding of FG2811.72 and MC631 mAbs to the glycan array was detected with goat anti-mouse IgG biotinylated followed by streptavidin-Cy3. The result confirmed the HSA-glycan ELISA result. FG2811.72 bound to SSEA-4 strongly and specifically whereas MC631 bound strongly to SSEA-3 and SSEA-4 glycans and; moderately to Globo-H glycan (Figure 6.9). Print buffer alone and biotinylated glycan were used as negative and positive controls (pc) respectively.

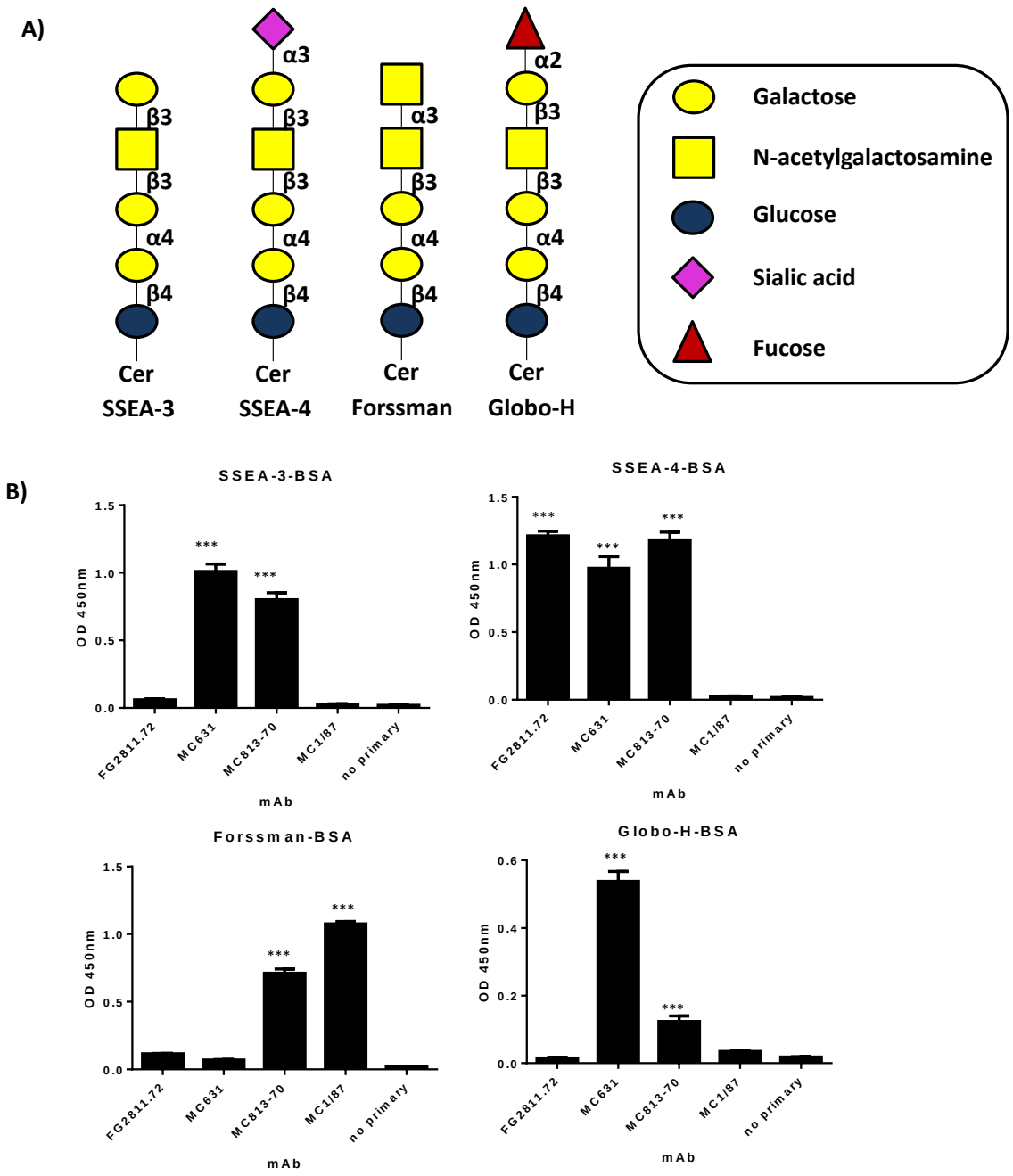


Figure 6.8: A) Chemical structure of the SSEA-3, SSEA-4, Forssman and Globo-H glycoepitopes and abbreviations of all glycoepitope analogs; B) Reactivity of the FG2811.72 and MC631 mAbs with commercial BSA coupled glycan antigens (SSEA-3, SSEA-4, Forssman and Globo-H). Binding of FG2811.72 and MC631 mAbs (3.6×10^{-8} M) to BSA coupled glycans was assessed by ELISA. Biotinylated anti-mouse IgG secondary antibody was used as negative control. Antibody binding was measured by absorbance at 405nm. Error bars represent the mean ± STD of quadruplicate wells (*, $P< 0.05$; **, $P<0.01$ and *** $P<0.001$ versus control, ANOVA followed by Bonferroni multiple comparisons test, GraphPad Prism 6).

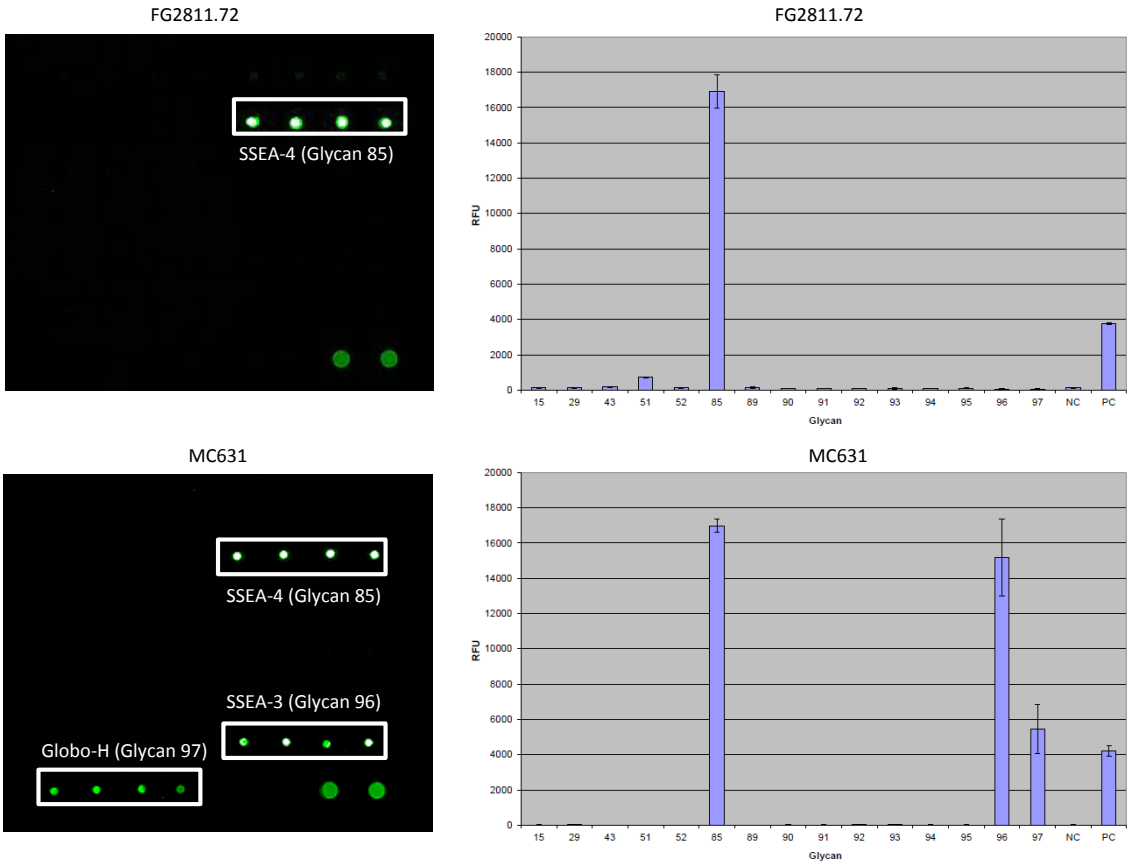


Figure 6.9: Binding of the FG2811.72 and MC631 mAbs to the Z Biotech, LCC glycan array. FG2811.72 and MC631 mAbs (3.6 x 10^{-7} M) were screened against the Z Biotech, LCC glycan array which is composed of 15 mammalian glycan targets, by ELISA. The fine specificity between FG2811.72 mAb and MC631 mAb were compared. *(Glycan 85: SSEA-4, Glycan 96: SSEA-3, Glycan 97: Globo-H)*.

Table 6.2: Glycan epitopes present on the Z Biotech, LCC glycan array.		
ID	**Glycan Name**	**Structure**
15	β-D-N-Acetylgalactosaminyl-1,3-galactose	GlaNAcβ-1,3-Gal
29	Globotriose	Galα-1,4-Galβ-1,4-Glc
43	P1 antigen	Galα-1,4-Galβ-1,4-GlcNAc
51	Sialyl Lewis x (SLe^x)	Neu5Acα-2,3-Galβ-1,4-(Fucα-1,3)-GlcNAcβ-1,3-Gal
52	Sialyl Lewis a (SLe^a)	Neu5Acα-2,3-Galβ-1,3-(Fucα-1,4)-GlcNAcβ-1,3-Gal
85	Stage-specific Embryonic Antigen 4 (SSEA-4)	Neu5Acα-2,3-Galβ-1,3-GalNAcβ-1,3-Galα-1,4-Galβ-1,4-Glc
89	Tn antigen	D-GalNAc
90	T antigen	Galβ-1,3-GalNAc
91	α-D-N-Acetylgalactosaminyl-1,3-galactose	GalNAcα-1,3-Gal
92	α-D-N-Acetylgalactosaminyl-1,3-galactose-β-1-4-glucose	GalNAcα-1,3-Galβ-1,4-Glc
93	B-1,6-galactobiose	Galβ-1,6-Gal
94	Forssman disaccharide	GalNAcα-1,3-GalNac
95	Receptor for pili of Pseudomonas aeruginosa	GalNAcβ-1,4-Gal
96	Stage-specific Embryonic Antigen 3 (SSEA-3)	Galβ-1,3-GalNAcβ-1,3-Galα-1,4-Galβ-1,4-Glc
97	Globo-H	Fucα-1,2-Galβ-1,3-GalNacβ-1,3-Galα-1,4-Galβ-1,4-Glc

It was then of interest to understand why the genetically engineered SSEA-3-LMTK cell line stimulated anti-SSEA-4 antibody response. SSEA-3-LTMK cell line was produced by transducing wild type LMTK cells with α-1-4-galactosyltransferase, β-1-3-N-acetylgalactosaminyltransferase and β-1-3-galactosyltransferase genes, which all together make SSEA-3 glycans. However, FG2811.72 recognises SSEA-4 glycan, which is similar to SSEA-3 in structure except that it has an additional terminal sialic acid moiety. Altogether, our results suggest that the LMTK wild type cell expressed α-2,3-sialyltransferase, which is an enzyme that transfers sialic acid to nascent oligosaccharides. To investigate whether LMTK cell expressed α-2,3-sialyltransferase, FG88.2 (anti-Lewis a; Le^a) and FG129 (anti-Sialyl-Lewis a; $SLea^)$ mAbs were used to screen for binding to G6B3R9 cells. As mentioned in the previous chapter, the G6B3R9 cell line was produced by transducing wild type LMTK cells with Lc3 synthase (B3gnt5), β-1,4-galactosyltransferase (β3Gal-T1) and α-1,4-fucosyltransferase (FUT3) genes to produce only the Le^a glycan. LMTK and HCT15 cell lines were included as negative and positive controls respectively. Secondary antibody alone (biotinylated anti-mouse IgG) and medium alone (RPMI) were used as negative controls (Figure 6.10). Both FG88.2 and FG129 mAbs showed strong binding to G6B3R9 (FG88.2 Gm: 137; FG129 Gm: 178) and HCT15 (FG88.2 Gm: 150; FG129 Gm: 247) cells at 3.6×10^{-8} M suggesting that LMTK cells do express sialyltransferase. Both mAbs did not bind to LMTK cells and the negative controls did not show any reactivity with any of the all cell lines used. Therefore, for the subsequent study, the SSEA-3-LMTK cell line is renamed as being a SSEA-3/4-LMTK cell line.

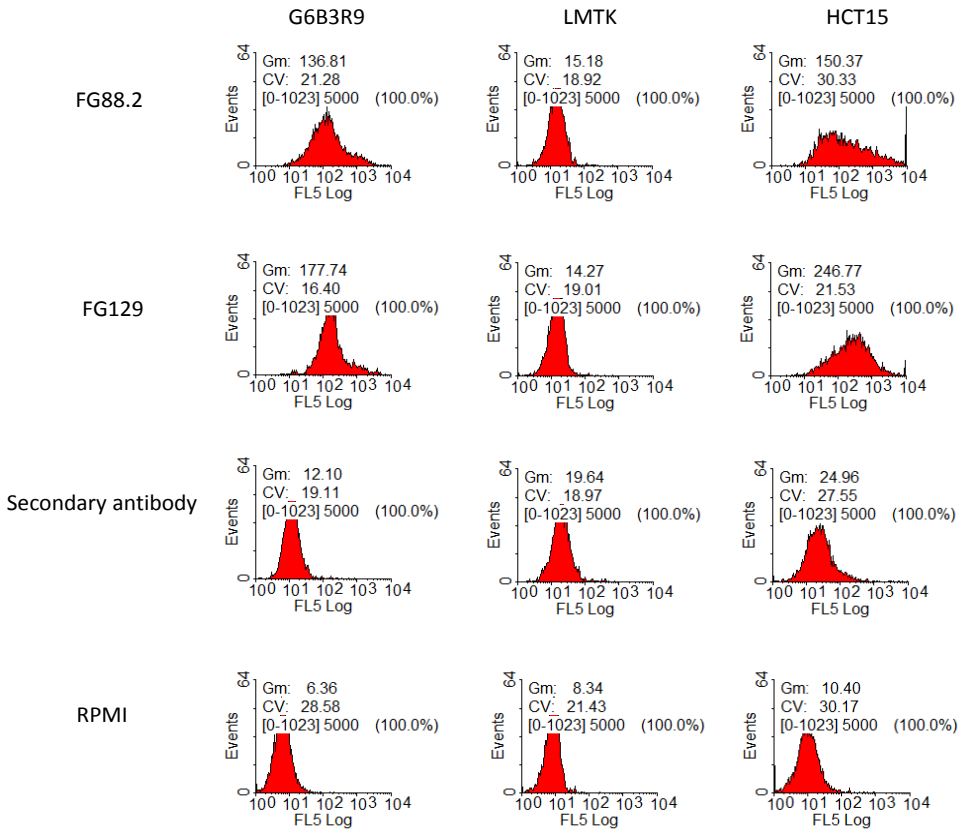


Figure 6.10: Binding of FG88.2 and FG129 mAbs to G6B3R9 (Le^a-expressing cell line), LMTK and HCT15 cell surface antigens. Binding of FG88.2 and FG129 mAbs (2.4 x 10^{-8} M) to G6B3R9, LMTK and HCT15 cells were assessed by indirect immunofluorescence staining and flow cytometric analysis. No primary/secondary antibody alone and medium alone (RPMI) were included as negative controls. Results are expressed as geometric mean values (Gm).

DNA sequencing (by Dr Richard McIntosh) revealed that FG2811.72 (Figure 6.11a and b) mAb belonged to the IGHV2-3*01 heavy chain and IGKV4-63*01 gene families. Assessment of the mutations showed 9 nucleotide differences between the FG2811.72 heavy chain and the germline sequence, with 5 changes in amino acid residues. Similarly, there were 7 nucleotide differences between the FG2811.72 kappa chain and the germline sequence, with 5 changes in amino acid residues as a result. The nature and pattern of mutations suggest somatic hypermutation and affinity maturation.

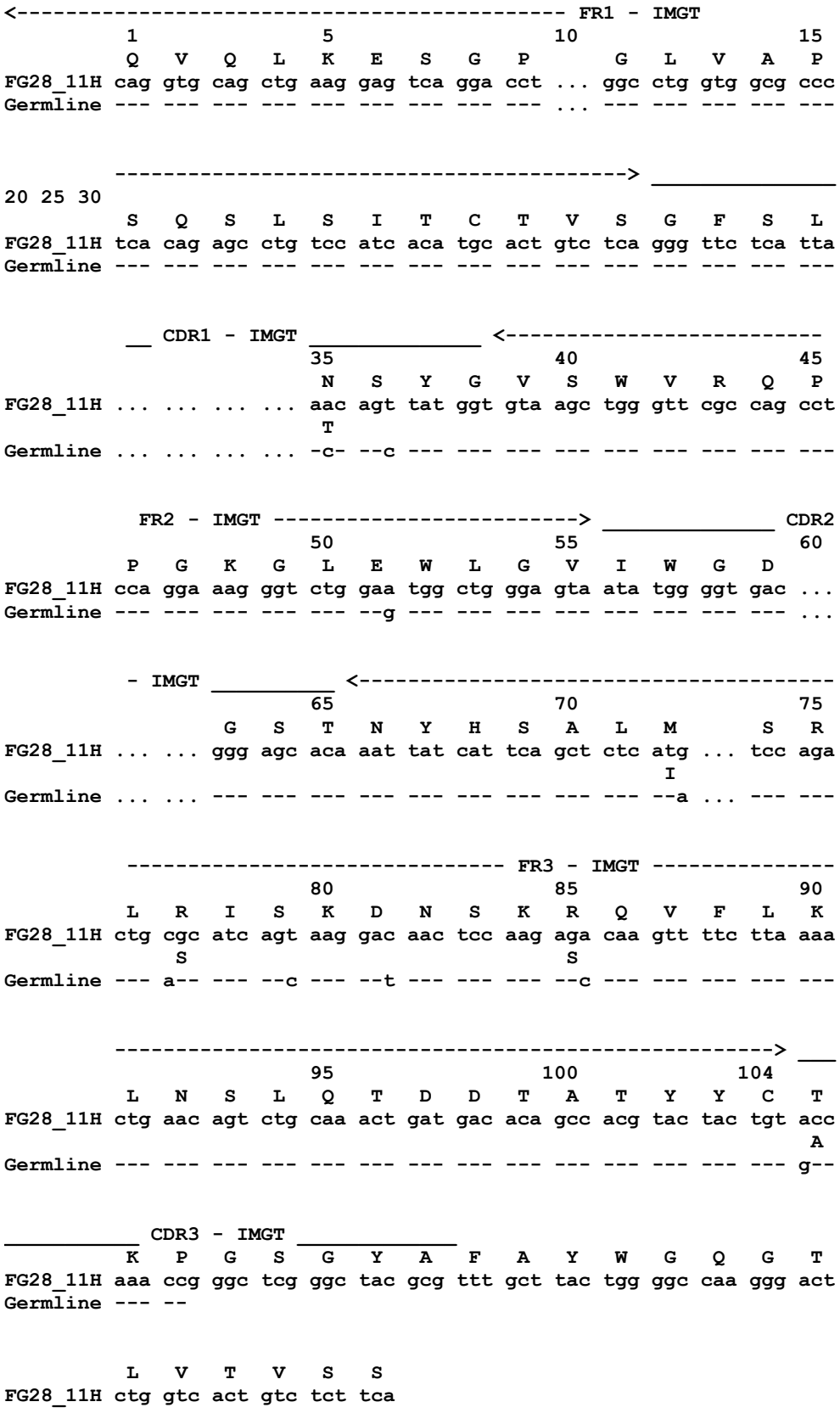


Figure 6.11a: Amino acid and nucleotide sequence of the FG2811.72 IgG3 heavy chain variable region. Nucleotide and amino acid sequence of the mature FG2811.72 heavy chain variable region, showing framework regions (FR) 1 to 3 and complementarity determining regions (CDR) 1 to 3.

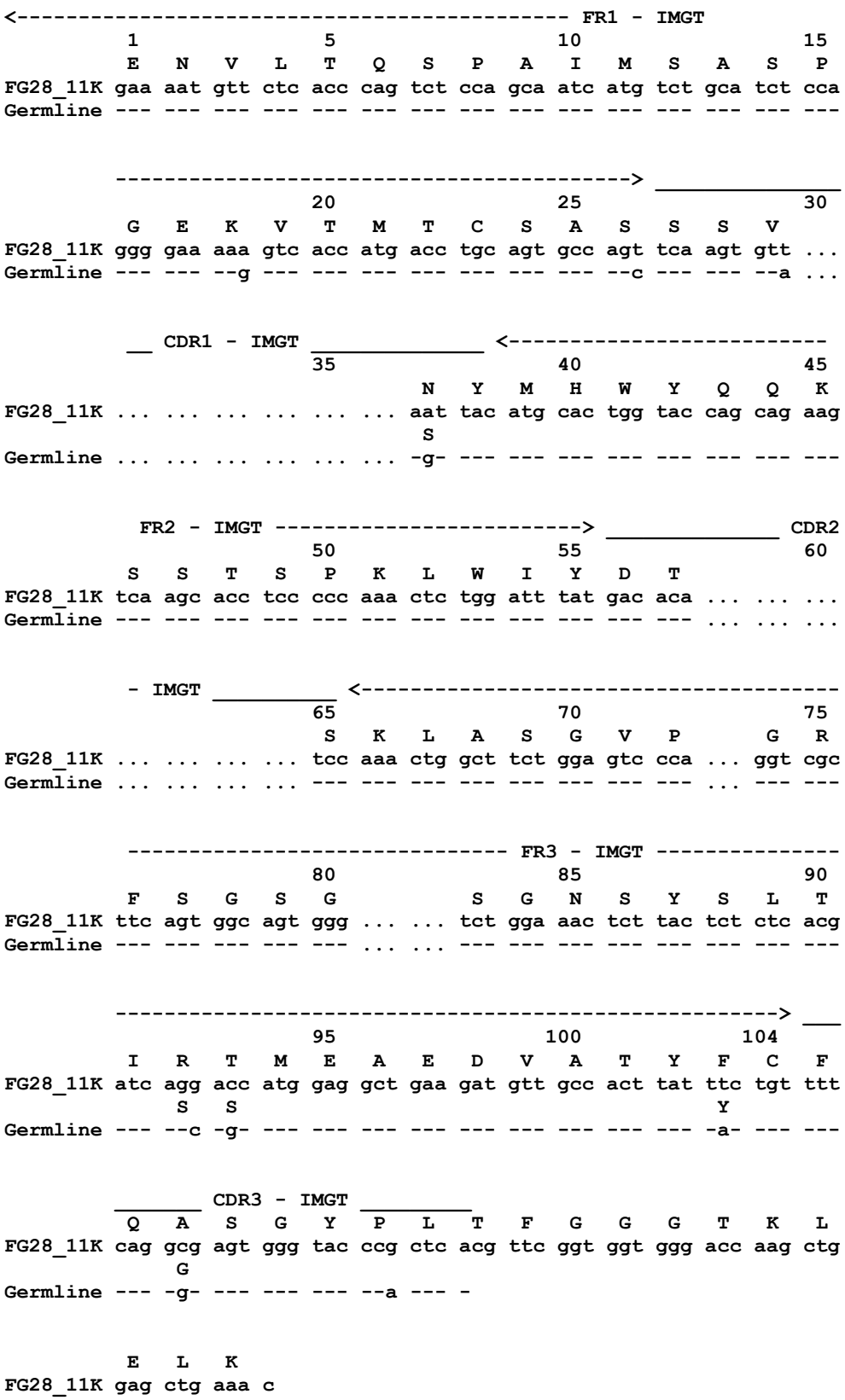


Figure 6.11b: Amino acid and nucleotide sequence of the FG2811.72 mouse kappa light chain variable region. Nucleotide and amino acid sequence of the mature FG2811.72 kappa chain variable

region, showing framework regions (FR) 1 to 3 and complementarity determining regions (CDR) 1 to 3.

6.2.3: FG2811.72 recognises SSEA-4 with high functional affinity

The SSEA-3/4-LMTK plasma membrane lipid antigen binding kinetics of FG2811.72 mAb was examined using SPR (Biacore X) (Dr Mireille Vankemmelbeke). Fitting of the binding curves revealed strong apparent functional affinity (K_d ~10^{-9} M) with fast association (~ 10^5 1/Ms) and slow dissociation (~10^{-4} 1/s) rates for FG2811.72. This was consistent with EC_{50} values derived from SSEA-3/4-LMTK plasma membrane lipid ELISA (EC_{50} = 6.8 x 10^{-10} M) (Figure 6.12) and cellular functional affinity (K_d = 5 x 10^{-9} M) (Figure 6.13).

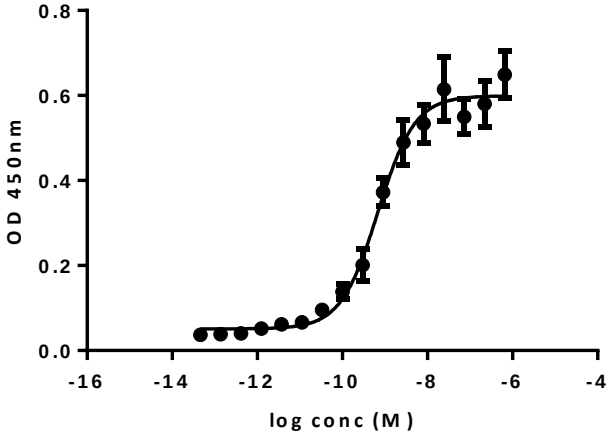


Figure 6.12: SSEA-3/4 plasma membrane lipid ELISA. A concentration range of the FG2811.72 mAb was incubated in SSEA-3/4 plasma membrane lipid - coated microwells. EC_{50} values (6.8 x 10^{-10} M) were obtained via non-linear regression on log-transformed data (GraphPad Prism 6).

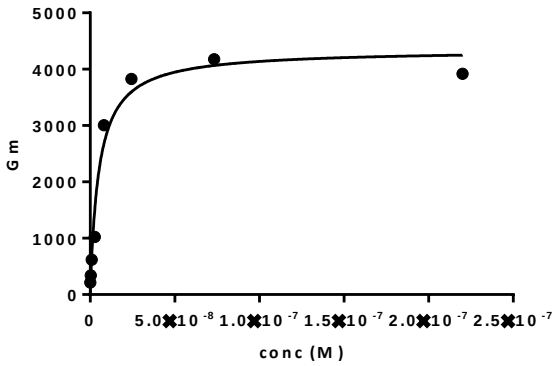


Figure 6.13: SSEA-3/4-LMTK cell surface binding. SSEA-3/4-LMTK cells were incubated with a concentration range of FG2811.72 mAb and cell binding analysed using flow cytometry. Fitting of the background - subtracted data to a one site specific binding model (GraphPad Prism 6) generated the K_d values (5 x 10^{-9} M).

6.2.4: Reduced FG2811.72 staining is associated with better breast cancer patient survival; and FG2811.72 staining is an independent prognostic marker

In order to assess the possible targets for FG2811.72 mAb, paraffin embedded breast and ovarian TMAs were stained. FG2811.72 stained 28% (223/798) of breast and 14% (41/289) of ovarian tumours. Examples of FG2811.72 staining level of breast and ovarian tumour tissues are shown (Figure 6.14). No significant association between FG2811.72 glycoepitope expression and clinicopathological variables was observed in ovarian tumour TMAs.

In contrast, in the breast cohort, FG2811.72 expression was positively correlated with ER status (P< 0.001), PgR status (P< 0.001), HER2 status (P< 0.001), EGFR (P< 0.001), P53 (P< 0.001) and BCL-2 (P <0.05) expression. FG2811.72 expression was negatively correlated with grade (P <0.001), tubule formation (P <0.01), mitosis (P <0.05), TNBC status (P <0.001), basal phenotype (P <0.001), CK5/6 (P <0.001), CK14 (P <0.001) and CK17 (P <0.01). Reduced expression of FG2811.72 glycoepitope was associated with poor prognosis (P = 0.013), ER positive subgroup that received Tamoxifen treatment (P = 0.032), recurrence (P = 0.001) and distant metastasis (P = 0.001) in breast cancer patients (Figure 6.15). On multivariate analysis using Cox regression, reduced FG2811.72 (P = 0.004) glycoepitope expression in breast cancer was a marker of good prognosis which was independent of tumour stage (P< 0.001), grade (P< 0.001) and size (P = 0.018).

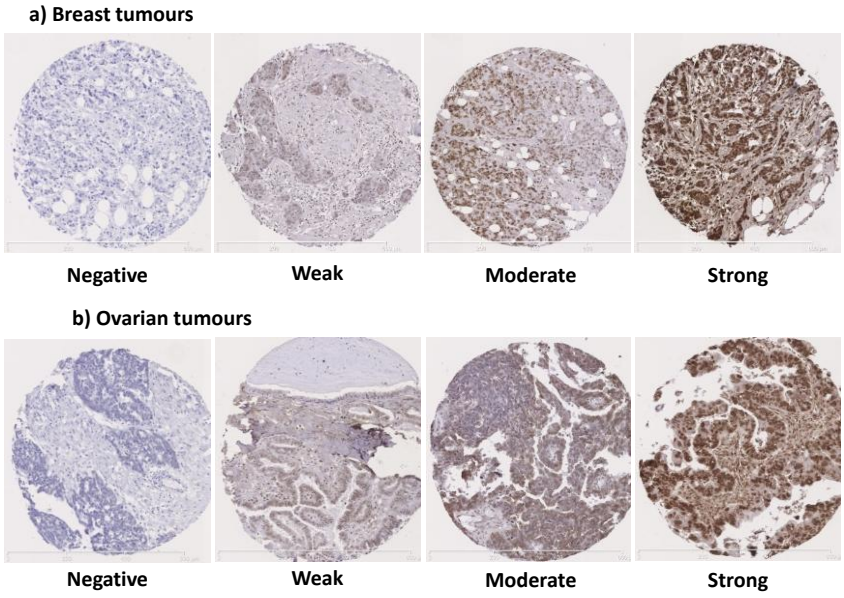


Figure 6.14: Breast and ovarian tumour tissues staining. Breast and ovarian tumour TMAs were stained with FG2811.72 (8.1 x 10^{-9} M). Representative images of negative, weak, moderate and strong FG2811.72 staining are shown (*magnification x20*).

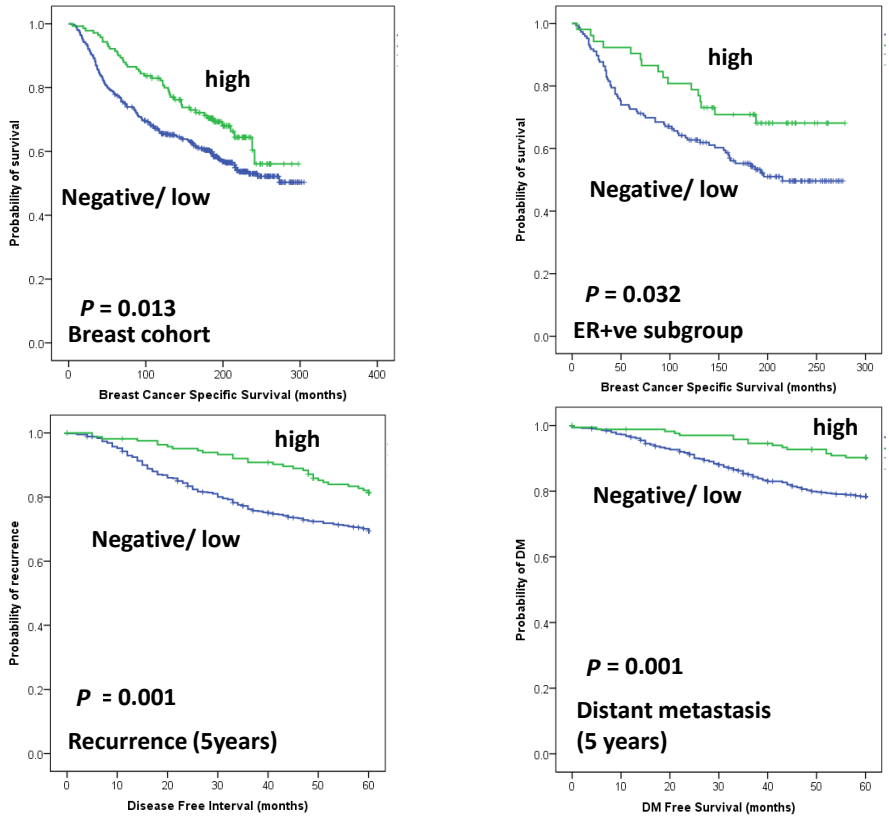


Figure 6.15: Survival analysis. Kaplan-Meier analysis of breast cancer specific survival in breast cancer TMA cohort showing the impact of FG2811.72 glycoepitope expression. Significance was determined using the log-rank test.

6.2.5: FG2811.72 mAb exhibits limited normal tissue cross reactivity

To assess the possible toxicity of FG2811.72 mAb, a normal human TMA (31 different normal human tissues) was stained at 8.1 x 10^{-9} M. MC813-70 (anti-SSEA-4) mAb was included as comparison at 8.1 x 10^{-9} M. FG2811.72 mAb did not stain placenta, esophagus, rectum, gall bladder, skin, adipose tissue, heart, skeletal muscle, bladder, ileum, spleen, brain, jejunum, stomach, breast, kidney, cerebellum, thymus, cervix, lung, colon, ovary, tonsil, diaphragm, uterus, duodenum and thyroid. Staining was seen against testis (moderate germ cell lining semiferous tubules nuclear staining), liver (weak bile duct staining) and pancreas (moderate to strong parenchymal, islet cell, ductal nuclear staining) (Figure 6.16a, b). MC813-70 did not stain liver, esophagus, rectum, gall bladder, skin, adipose, heart, skeletal muscle, bladder, IIeum, spleen, jejunum, stomach, cerebellum, thymus, lung, colon, ovary, tonsil, diaphragm, uterus, duodenum and thyroid. MC813-70 stained more normal human tissues compared to FG2811.72 mAb. MC813-70 stained placenta (weak nuclear staining), bladder (weak bladder lining cytoplasm and nuclear staining), brain (weak nuclear staining), testis (moderate germ cell lining semiferous tubules nuclear staining), kidney (weak to moderate tubules and glomerulus nuclear staining), breast (moderate to strong macrophage cell like staining), cervix (moderate to strong macrophage cell like staining) and pancreas (moderate to strong parenchymal,

islet cell, ductal nuclear staining) (Figure 6.16c). The comparison between FG2811.72 and MC813-70 staining on normal human tissues was summarised in Table 6.3.

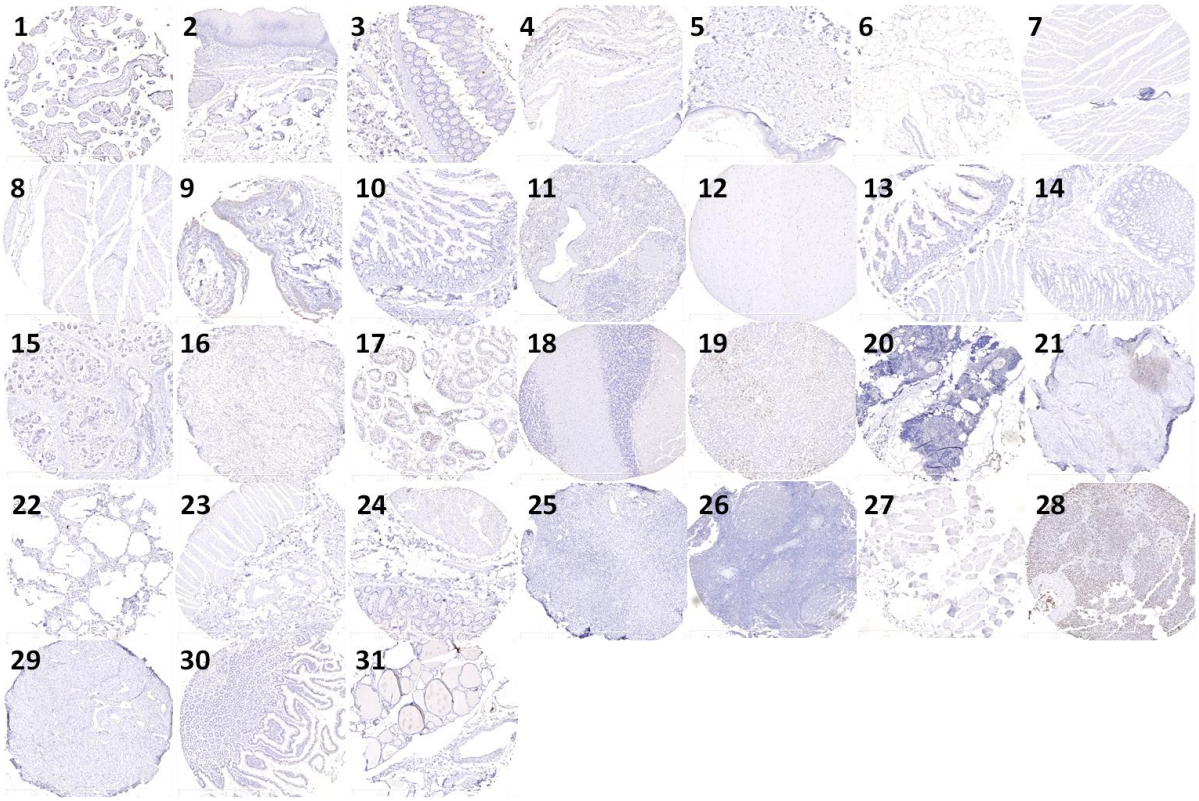


Figure 6.16a: Paraffin embedded normal human tissue staining. AMSBIO paraffin embedded normal human tissue microarray was stained by ABC-immunohistochemistry with FG2811.72 (8.1 x 10^{-9} M) counterstained by hematoxylin. 1) placenta, 2) esophagus, 3) rectum, 4) gall bladder, 5) skin, 6) adipose tissue, 7) heart, 8) skeletal muscle, 9) bladder, 10) ileum, 11) spleen, 12) brain, 13) jejunum, 14) stomach, 15) breast, 16) kidney, 17) testis, 18) cerebellum, 19) liver, 20) thymus, 21) cervix, 22) lung, 23) smooth muscle, 24) colon, 25) ovary, 26) tonsil, 27) diaphragm, 28) pancreas, 29) uterus, 30) duodenum, 31) thyroid (*magnification x20*).

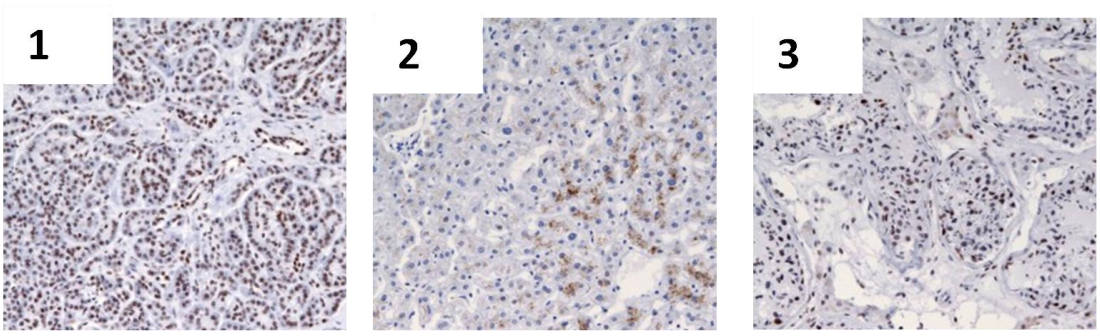


Figure 6.16b: Subcellular location of FG2811.72 glycoepitopes on representative paraffin embedded human normal tissues. AMSBIO paraffin embedded normal human tissue microarray was stained by ABC-immunohistochemistry with FG2811.72 (8.1 x 10^{-9} M) counterstained by hematoxylin. 1) pancreas (moderate parenchymal, islet cell and ductal nuclear staining), 2) liver (weak bile duct staining) and 3) testis (moderate germ cell lining semiferous tubules nuclear staining) (*magnification ×20*).

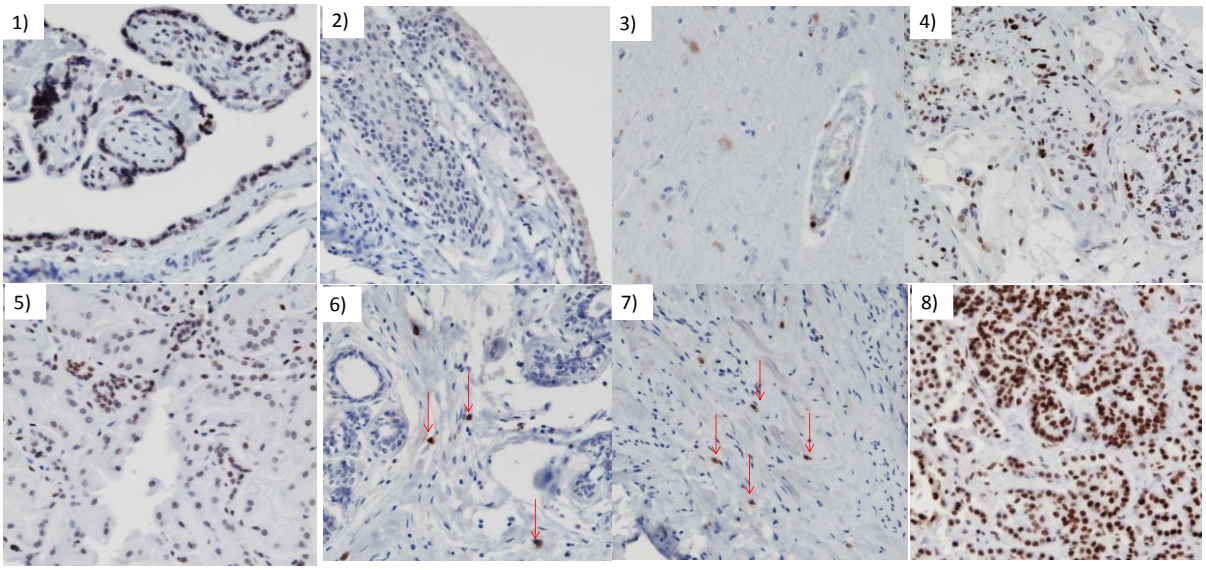


Figure 6.16c: Subcellular location of MC813-70 (anti-SSEA-4) mAb glycoepitopes on representative paraffin embedded human normal tissues. AMSBIO paraffin embedded normal human tissue microarray was stained by ABC-immunohistochemistry with MC813-70 (8.1 x 10^{-9} M) counterstained by hematoxylin. 1) Placenta (weak nuclear staining), 2) bladder (weak bladder lining cytoplasm and nuclear staining), 3) brain (weak nuclear staining), 4) testis (moderate germ cell lining semiferous tubules nuclear staining), 5) kidney (weak to moderate tubules and glomerulus nuclear staining), 6) breast (moderate to strong macrophage cell like staining), 7) cervix (moderate to strong macrophage cell like staining) and 8) pancreas (moderate to strong parenchymal, islet cell, ductal nuclear staining) (*magnification ×20*).

Tissue	FG2811.72	MC813-70
Placenta	0	Weak nuclear staining (1)
Esophagus	0	0
Rectum	0	0
Gall bladder	0	0
Skin	0	0
Adipose	0	0
Heart	0	0
Skeletal muscle	0	0
Bladder	0	Weak bladder lining cytoplasm and nuclear staining (1)
Illeum	0	0
Spleen	0	0
Brain	0	Weak nuclear staining (1)
Jejunum	0	0
Stomach	0	0
Breast	0	Moderate to strong macrophage cell like staining (2/3)
Kidney	0	Weak to moderate tubules and glomerulus nuclear staining (1/2)
Testis	Moderate germ cell lining semiferous tubules nuclear staining (2)	Moderate germ cell lining semiferous tubules nuclear staining (2)
Cerebellum	0	0
Liver	Weak bile duct staining (1)	0
Thymus	0	0
Cervix	0	Moderate to strong macrophage cell like staining (2/3)
Lung	0	0
Small intestine	0	0
Colon	0	0
Ovary	0	0
Tonsil	0	0
Diaphragm	0	0
Pancreas	Moderate parenchymal, islet cell and ductal nuclear staining (2)	Moderate to strong parenchymal, islet cell, ductal nuclear staining (2/3)
Uterus	0	0

Table 6.3: Paraffin embedded normal human tissue microarray staining by FG2811.72 mAb and MC813-70. Paraffin embedded normal human tissue TMA (AMSBIO) was stained with FG2811.72 and MC813-70 at the antibody concentration of 8.1 x 10^{-9} M. The staining intensity was scored on subcellular location of FG2811.72 glycoepitopes on representative paraffin embedded human normal tissues according to: negative (0), weak (1), medium (2) and strong (3).

6.2.6: FG2811.72 mAb does not bind to erythrocytes

SSEA-3 and SSEA-4 have been reported to be present on the erythrocytes of the majority of the population as defined by MC631 and MC813-70 mAbs (Tippett, Andrews, Knowles, Solter, & Goodfellow, 1986). Therefore, binding of FG2811.72 mAb (7.3 x 10^{-8} M) to erythrocytes from 5 healthy donors was assessed by flow cytometry (Figure 6.17). The anti-CD55 mAb (791T/36; 7.3 x 10^{-8} M; Gm: 202) was included as positive control whereas an IgG isotype and PBS were used as negative controls. FG2811.72 (Gm: 10) did not bind to erythrocytes from any of the 5 donors tested. An erythrocyte agglutination assay further confirmed that FG2811.72 mAb (4.5 x 10^{-9} M to 7.3 x 10^{-8} M) did not agglutinate erythrocytes from the 5 healthy donors. In contrast, 791T/36 agglutinated erythrocytes from all donors. PBS was used as the negative control (Figure 6.18).

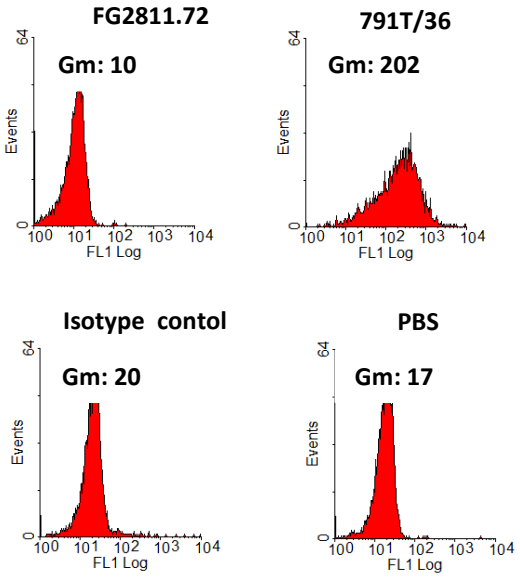


Figure 6.17: Evaluation of FG2811.72 mAb binding to erythrocytes by flow cytometry. Erythrocyte binding by FG2811.72 (7.3 x 10-8 M) mAb was compared to anti-CD55 positive control mAb (791T/36; 7.3 x 10^{-8} M) by flow cytometry. IgG isotype control and medium alone were negative controls. Result representatives of 5 normal donors.

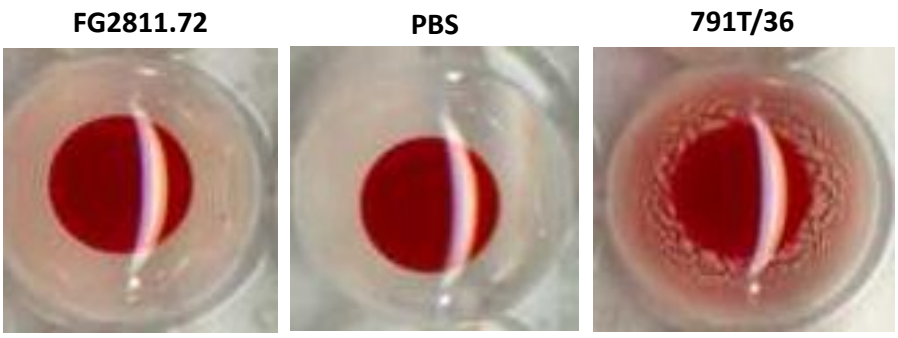


Figure 6.18: Hemagglutination assay. Erythrocytes agglutination by FG2811.72 mAb (3.6×10^{-8} M) was compared to anti-CD55 positive control mAb [791T/36; (3.6×10^{-8} M)]. PBS was used as the negative control. Results are representative of 5 donors.

6.2.7: FG2811.72 mAb as therapy for breast and ovarian cancers

6.2.7.1: FG2811.72 mAb inhibits SSEA-3/4-LMTK cell growth and induces potent immune mediated cytotoxicity and internalises into tumour cells efficiently

It is suggested in the literature that glioblastoma multiforme overexpresses SSEA-4 glycolipid as defined by the MC813-70 mAb. Thus, a panel of glioblastoma cell lines were screened for SSEA-4 expression, using both FG2811.72 and MC813-70 at 3.6×10^{-8} M by flow cytometry analysis (Figure 6.19a). Mouse IgG3 kappa isotype control and medium alone (RPMI) were used as negative controls. U251 and U87 are adult glioblastoma multiforme (GBM) cell lines, SF188 and KNS42 are paediatric GBM and UW2283 and DAOY are medulla blastoma cell lines. FG2811.72 bound to UW2283 (Gm: 36) and DAOY (Gm: 50) weakly but failed to bind to the other cell lines. In contrast, MC813-70 bound to UW2283 (Gm: 152) and DAOY (Gm: 169) strongly. Similar to FG2811.72, MC813-70 did not bind to the rest of the cell lines.

Next, binding of the FG2811.72 mAb (3.6×10^{-8} M) to a panel of other tumour cell lines (Figure 6.19b) was studied by flow cytometry. W6/32 (anti-HLA, B and C) and secondary alone (biotinylated anti-mouse IgG antibody) were used as positive and negative controls respectively. FG2811.72 bound strongly to SKOV3 (SKOV3 Gm: 203; W6/32 Gm: 146; secondary alone Gm: 18), moderately to T47D (T47D Gm: 95; W6/32 Gm: 653; secondary alone Gm: 28); OVCAR5 (OVCAR5 Gm: 86; W6/32 Gm: 439; secondary alone Gm: 23) and MCF7 (MCF7 Gm: 76; W6/32 Gm: 200; secondary Gm: 33); weakly to IGROV1 (IGROV1 Gm: 41; W6/32 Gm: 419; secondary alone Gm: 21); and failed to bind DU4475 (DU4475 Gm: 26; W6/32 Gm: 514; secondary alone Gm: 23), HCC1187 (HCC1187 Gm: 22; W6/32 Gm: 907; secondary alone Gm: 20), Colo205 (Colo205 Gm: 13; W6/32 Gm: 83; secondary alone Gm: 13) and HCT15 (HCT15 Gm: 22; W6/32 Gm: 24; secondary alone Gm: 22) cell lines. W6/32 failed to bind to HCT15, indicating that HCT15 does not express HLA A, B and C.

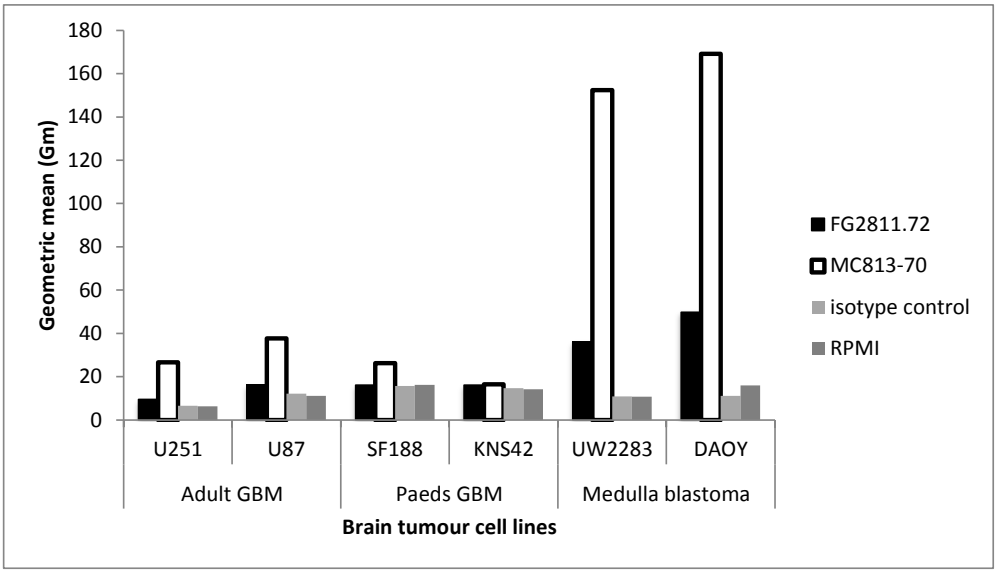


Figure 6.19a: FG2811.72 and MC813-70 tumour cell binding determined by flow cytometry. FG2811.72 and MC813-70 binding to a panel of tumour cell line at 3.6 x 10^{-8} M was assessed by flow cytometry and presented as Gm values. Mouse IgG3 kappa isotype control and medium alone (RPMI) were used as negative controls.

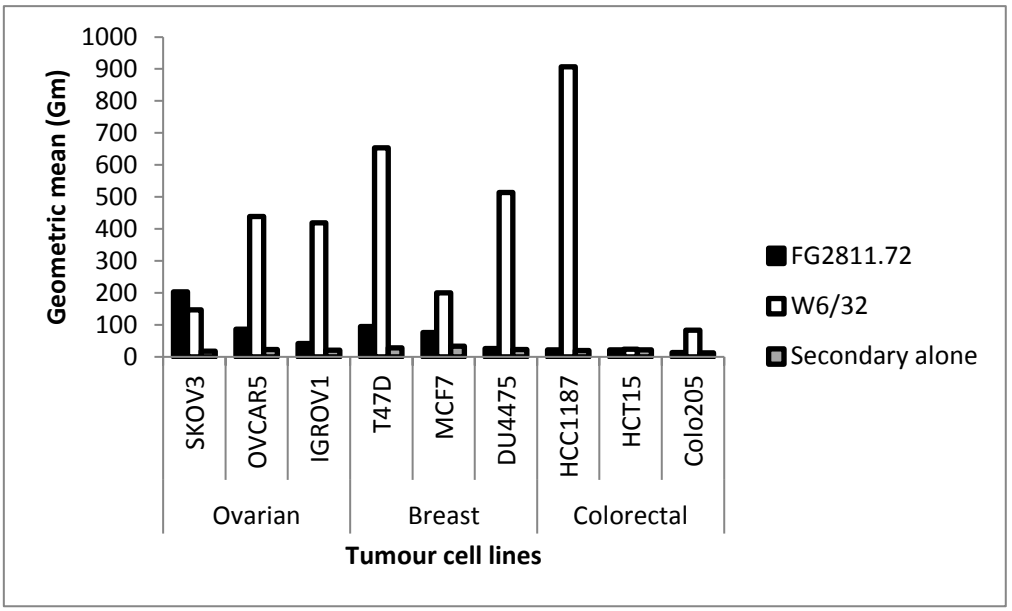


Figure 6.19b: FG2811.72 tumour cell binding determined by flow cytometry. FG2811.72 binding to a panel of tumour cell line at 3.6 x 10^{-8} M was assessed by flow cytometry and the results presented as Gm values. W6/32 (anti-HLA, B and C) and secondary alone (biotinylated anti-mouse IgG antibody) were included as positive and negative controls respectively.

SKOV3 and T47D cells were selected as high and moderate FG2811.72 antigen expression cells respectively for subsequent cytotoxicity assays. The ability of FG2811.72 mAb to induce tumour cell death through ADCC was investigated. Human PBMCs were used as the source of effector cells while SKOV3 and T47D cells served as target cells. Anti-CD55 mAb (791T/36) was used as the negative

control. The number of cells killed by FG2811.72 mAb was measured after 18 hr incubation at 37^0C (Figure 6.20). SKOV3 (EC_{50}: 10^{-10} M) was susceptible to FG2811.72 mAb killing in a concentration dependent manner showing a maximum of 66% lysis. FG2811.72 mAb failed to induce T47D cell killing via ADCC.

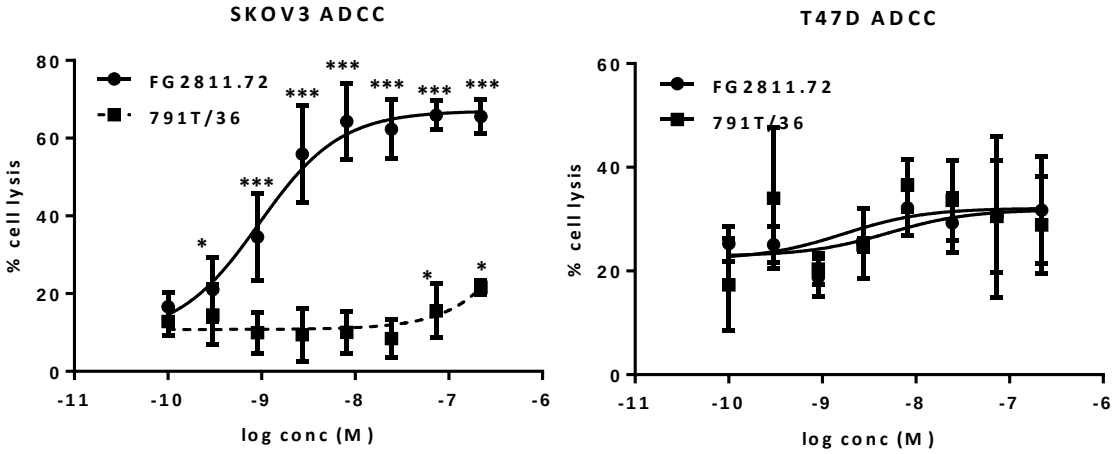


Figure 6.20: ADCC killing of tumour cells by FG2811.72 mAb. Dose-dependent ADCC activity of FG2811.72 mAb on SKOV3 and T47D cells. The ^{51}Cr-labeled cells were co-incubated with increasing concentrations (3.3 x 10^{-11} to 2.2 x 10^{-7} M) of FG2811.72 mAb and human PBMCs (E:T of 100:1).^{51}Cr released into the supernatant was measured and expressed as the percentage of total ^{51}Cr released with 10% Triton-X. 791T/36 mAb (3.3 x 10^{-11} to 2.2 x 10^{-7} M) was used as the negative control mAb. Significance versus PBMC control was established by ANOVA followed by Bonferroni multiple comparisons test, GraphPad Prism 6. (*, P< 0.05; **, P<0.01 and *** P<0.001 versus control; n=3).

CDC is known to be an important mechanism involves in eliminating tumour cells *in vivo*. The capacity of the SKOV3 and T47D cells to be killed by CDC induced by FG2811.72 mAb in the presence or absence of human serum as source of complement was assayed (Figure 6.21). Anti-CD55 mAb (791T/36) was used as negative control mAb. FG2811.72 mAb showed a maximum of 48% lysis of SKOV3 (EC_{50}: 10^{-9} M). Again, FG2811.72 failed to induce T47D cell killing via CDC.

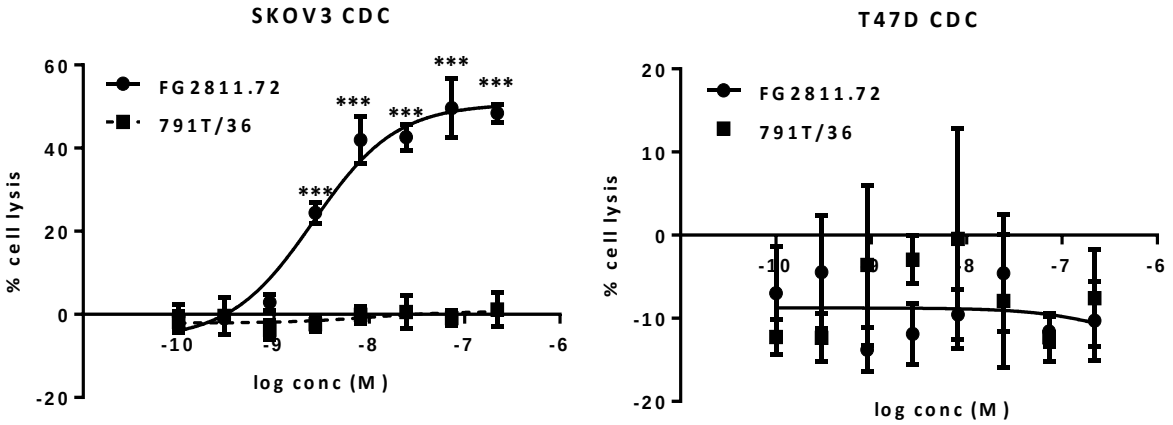


Figure 6.21: CDC killing of tumour cells by FG2811.72 mAb. Dose-dependent CDC activity of FG2811.72 mAb on SKOV3 and T47D cells. The ^{51}Cr-labeled cells were co-incubated with increasing concentrations (3.3 x 10^{-11} to 2.2 x 10^{-7} M) of FG2811.72 mAb and human serum.^{51}Cr released into the supernatant was measured and expressed as the percentage of total ^{51}Cr released with 10% Triton-X. 791T/36 mAb (3.3 x 10^{-11} to 2.2 x 10^{-7} M) was used as the negative control. Significance versus serum control was established by ANOVA followed by Bonferroni multiple comparisons test, GraphPad Prism 6. (*** *P*<0.001 versus control; n=3).

To check whether FG2811.72 induced direct cancer cell death, it was incubated with SSEA-3/4-LMTK cells at 37^{o}C for 2 hr. Hydrogen peroxide (H_2O_2) and medium alone (RPMI) were used as the positive and negative controls respectively. FG2811.72 mAb induced 74.7% and 59.3% PI uptake at 2.2 x 10^{-7} M and 7.3 x 10^{-8} M (Figure 6.22a). To determine whether FG2811.72 mAb inhibits tumour cell growth, the cell viability of SSEA-3/4-LMTK and SKOV3 cells treated with FG2811.72 mAb at 2.2 x 10^{-7} M was evaluated under a light microscope (Figure 6.22b). LMTK wild type cells and cells treated with medium alone (RPMI) were used as negative controls. SSEA-3/4-LMTK cells were observed to aggregate within seconds after the FG2811.72 mAb was added. However, this phenomenon did not develop when SKOV3 and LMTK cells were incubated with FG2811.72 mAb. FG2811.72-treated SSEA-3/4-LMTK and SKOV3 cells showed evidence of a decrease in cell numbers after 72 hr of mAb addition. FG2811.72 mAb showed no effect on LMTK cells. Cells incubated with medium alone did not show growth inhibition and achieved 100% confluency with some cell death over the 72 hr incubation period.

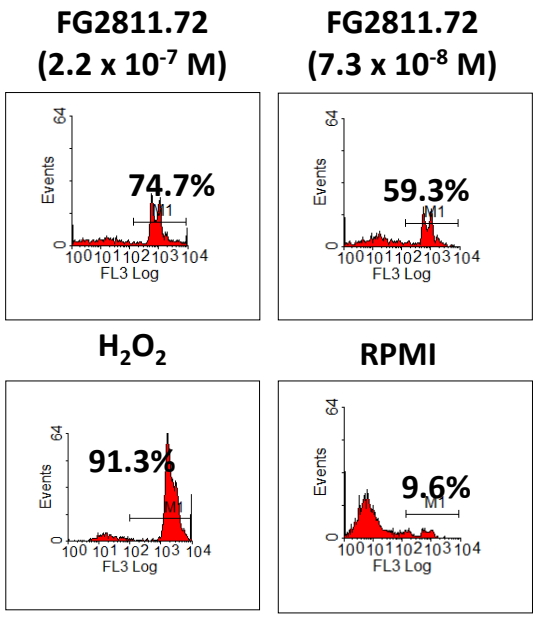


Figure 6.22a: FG2811.72 mAb induced direct cell death of SSEA-3/4-LMTK cells at 37^{0}C. PI uptake following mAb exposure was assessed by flow cytometric analysis. SSEA-3/4-LMTK cells were incubated with 2.2 x 10^{-7} M and 7.3 x 10^{-8} M of FG2811.72 mAb at 37^{0}C. Hydrogen peroxide (H_2O_2) and medium alone (RPMI) were included as positive and negative controls respectively.

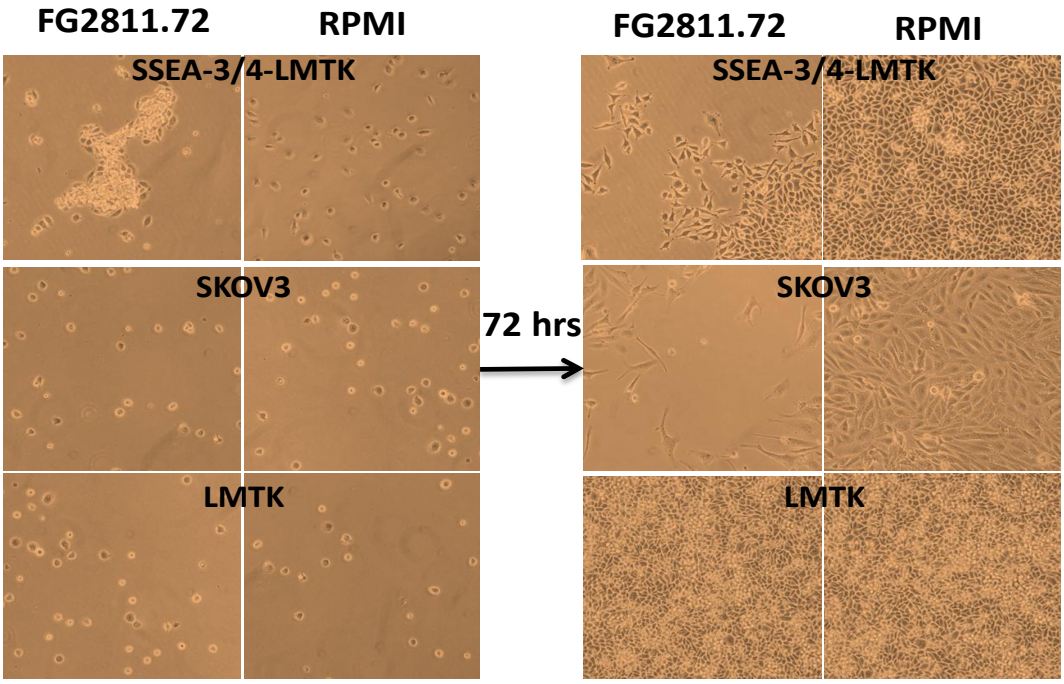


Figure 6.22b: Phase-contrast imaging of FG2811.72-treated tumour cells. Images (*magnification x10*) showing SSEA-3/4-LMTK, SKOV3 and LMTK cells after incubation with FG2811.72 (2.2 x 10^{-7} M) and medium alone (RPMI; negative control) for 72 hr.

To assess whether FG2811.72 mAb can be used as an antibody drug conjugate (ADC), internalisation of the FG2811.72 mAb into tumour cells was validated through the toxicity of a Fab-ZAP-FG2811.72 immune complex containing saporin. Internalisation of the Fab-ZAP-FG2811.72 complex, but not the Fab-ZAP alone, led to a dose dependent (IC_{50} ~10^{-9} M) decrease in cell viability of the high glycol-epitope expressing SKOV3 cells (Figure 6.23).

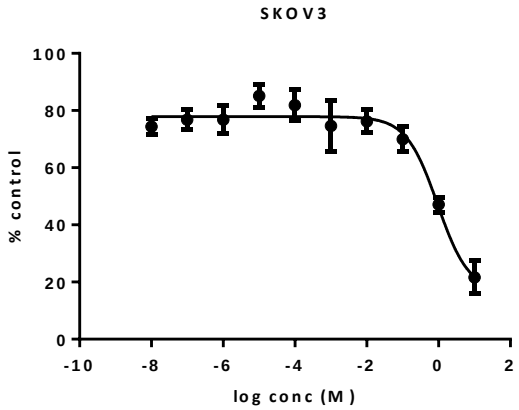


Figure 6.23: Targeted toxin (saporin) delivered by internalised FG2811.72 mAb in SKOV3 cells. The anti-proliferative effect of internalised FG2811.72 mAb pre-incubated with a saporin-linked anti-mouse IgG Fab fragment was evaluated using ^{3}H-thymidine incorporation. Concentrations refer to the FG2811.72 mAb. Results are presented as a percentage of proliferation of cells treated with primary mAbs only; normalised values are shown for SKOV3. Non-linear regression was performed using GraphPad Prism 6.

6.3: Discussion

Stage-specific embryonic antigens (SSEAs) have been widely used as markers to monitor the differentiation of mouse and human embryonic stem (ES) cells as well as their malignant counterparts, embryonic carcinoma (EC) cells (Henderson et al., 2002). Human inner cell mass (ICM), ES and EC cells have been reported to express SSEA-3 and SSEA-4 but lack of SSEA-1, differing from that of murine ICM, ES and EC cells, which express SSEA-1 but lack of SSEA-3 and SSEA-4 (Andrews, 1998; Draper, Pigott, Thomson, & Andrews, 2002; Gooi et al., 1981; Solter & Knowles, 1978). However, SSEA-3 and SSEA-4 antigens are downregulated during the differentiation of human ES cells (Draper et al., 2002). In murine, SSEA-3 and SSEA-4 are only expressed by unfertilised eggs and early cleavage embryos (Kannagi et al., 1983; Shevinsky et al., 1982).

In this study, we described a novel strategy to generate mAbs against SSEA-antigens that completely bypasses the need for adjuvants. The approach utilises stably transduced mouse fibroblast cells expressing SSEA-3 antigens on their cell surface [generous gift from Dr Emili Cid and Dr Fumiichiro

Yamamoto (IJC Josep Carreras Leukemia Research Institute, Barcelona)] for immunisation of mice. The LMTK mouse fibroblast cell line was chosen due to an undetected mutation which results in LMTK wild type cells expressing lactosylceramide glycolipid (Cid et al., 2013). In addition, SSEA-antigens are absence in adult mice, therefore it is possible to induce high immune response against SSEA-antigens on the cell surface of SSEA-3-LMTK cells in mice. Moreover, by immunising mice with mouse fibroblast cells, immune response against murine cell surface antigens would be reduced due to immune tolerance to self-antigens.

FG2811.72 mAb is a mouse IgG3 mAb, generated from mice immunised with SSEA-3-LMTK cells. Interestingly, rather than SSEA-3, FG2811.72 mAb recognised SSEA-4 specifically. This raised the possibility that LMTK cells may express α-2,3-sialyltransferase (encoded by ST3GAL2 gene) (Saito et al., 1997) , which is the major SSEA-4 synthase (Aloia et al., 2015; Saito et al., 1997) that transfers sialic acids to the SSEA-3 oligosaccharide chains. Further investigation on the binding of FG129 mAb (anti-SLe^a) to G6B3R9 cells confirmed that wild type LMTK cells express sialyltransferases. In a study, Lou and coworkers examined the expression of SSEA-3 and SSEA-4 on 134 cancer cell lines. SSEA-4 was observed to be expressed in 96 of the 134 cancer cell lines and SSEA-3 was found to be expressed mainly in cell lines with high SSEA-4 expression indicating that once SSEA-3 is synthesised, it is converted efficiently into SSEA-4 (Lou et al., 2014). RT-PCR analysis of the expression of sialyltransferases in LMTK wild type cell will be able to answer whether LMTK cells express the α-2,3-sialyltransferase.

In humans, the oligosaccharide chains of glycoproteins and glycolipids are often decorated by sialic acids, which are mediated by different sialyltransferases. Different sialyltransferases may establish different types of linkages [linkage through an α2-3- or an α2-6-bond to galactose (Gal); through an α2-6-bond to N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc); or through an α2-8-bond to another sialic acid, forming polysialic acid](Harduin-Lepers et al., 2001). In cancers, the aberrant expression of sialyltransferases often result in the overexpression of heavily sialylated glycans on the tumour cell surface (S. Hakomori, 2002); their expression is thought to mediate tumour progression, including invasion and metastasis (Chandrasekaran, Davila, Nixon, Goldfarb, & Mendicino, 1983; Egan, Coligan, Pritchard, Schnute, & Todd, 1976; Sawada, Lowe, & Fukuda, 1993) due to the fact that they can be specifically bound by cell adhesion molecules, such as selectins (Dimitroff et al., 1999). Overexpression of Sialyl Le^a (SLe^a) antigen has been reported in human colorectal, gastric, pancreatic and gall bladder adenocarcinomas (Atkinson et al., 1982; Koprowski et al., 1979; S. Sakamoto et al., 1989). Clinicopathological studies revealed that the high expression of SLe^a is a prognostic factor of colorectal cancer and is significantly associated with high incidence of

recurrence and the patient survival period (Nakayama, Watanabe, Katsumata, Teramoto, & Kitajima, 1995; Shimono, Mori, Akazawa, Adachi, & Sgimachi, 1994; Yamada et al., 1997). Importantly, Isozaki et al. demonstrated that high expression of SLe^a is significantly related with the presence of hepatic metastatic tumours (Isozaki, Ohyama, & Mabuchi, 1998). Like SLe^a, SLe^x has been reported to be involved in the cancer metastasis of a broad range of cancers, including lung (Fukuoka, Narita, & Saijo, 1998), breast (Julien et al., 2011), renal cell carcinoma (Tozawa et al., 2005) and bladder cancers (Matsusako, Muramatsu, Shirahama, Muramatsu, & Ohi, 1991), via the interaction with endothelial cells via E-selectin (Tozawa et al., 2005).

SSEA-4 is similar to SSEA-3 in terms of its structure, except that it has an additional terminal sialic acid residue. α-2,3-sialyltransferase, which is encoded by the ST3GAL2 gene, has been suggested to be the main enzyme to contribute to the sialylation of SSEA-3 into SSEA-4. SSEA-4 expression in a broad range of cancers has been assessed. In solid tumours, the overexpression of SSEA-4 has been found on glioblastoma (~55% of grade I, ~55% of grade II, ~60% of grade III and ~69% of grade IV astrocytoma) (Lou et al., 2014), renal cell carcinomas (Saito et al., 1997), breast cancer and breast cancer stem cells (Huang et al., 2013), basaloid lung cancer (Gottschling et al., 2013), epithelial ovarian carcinoma (Ye, Li, Hu, Zhou, Hu, et al., 2010), and oral cancer (Noto et al., 2013). The relationship between the SSEA-4 expression level and tumour malignancy varies in different cancers: High expression of SSEA-4 in basaloid lung cancer is associated with poor prognosis (Gottschling et al., 2013), whereas low expression SSEA-4 correlates with more advanced tumour stage and tumour cell differentiation in ovarian cancer (Ye, Li, Hu, Zhou, & Chen, 2010). In addition, higher expression of SSEA-4 appears to be a trend of association with higher grade of astrocytoma (Lou et al., 2014).

In this study, immunohistochemical analysis of a large cohort of patients with primary invasive breast carcinoma shows that FG2811.72 expression is positively associated with hormone receptors (ER, PgR and HER2) and reduced expression of FG2811.72 is significantly associated with poor outcome in the breast cohort, Tamoxifen treated ER positive subgroup, 5 years recurrence and 5 years distant metastasis of breast cancer patients, suggesting FG2811.72 could be useful as second line treatment targeting hormone receptor positive breast cancers, or tamoxifen resistant breast cancers. Importantly, this study is the first to demonstrate independent prognostic value for SSEA-4 expression in breast cancer as defined by FG2811.72 mAb.

In many human tumours, cancer stem cells (CSCs) have been identified and they represent approximately 10% of all tumour cells. They share many characteristics of the normal stem cells, such as the ability to renew and metabolically or physically protect themselves from xenobiotics and DNA damage. CSCs have been reported to be resistant to radiotherapy, primarily due to slow cell

cycling, lower proliferation, increased expression of DNA repair and anti-apoptotic genes (Deonarain, Kousparou, & Epenetos, 2009). Apart from radiotherapy resistance, CSCs have been reported to contribute to chemotherapy resistance as well. In normal stem cells, ABC-B1/ MDR1 and ABC-G2 pumps protect them from xenobiotic molecules. However, these pumps are also expressed on CSCs, which increased the ability of the CSCs to expel chemotherapy drugs such as daunorubicin and mitoxanthrone (Wulf et al., 2001). All these properties may explain why CSCs are resistant to therapy and lead to cancer metastasis and tumour relapse in cancer patients due to tumour cell regrowth after radiotherapy. The concept of targeting CSCs in cancer therapy has gained extensive attention especially antibody-directed therapies. However, the limitations are that compared to conventional antibody therapies, CSCs are usually present at low levels and the CSCs markers overlap with normal stem cells (Y. M. Yang & Chang, 2008). CD44 is the most established and common CSC marker. It is a 90 kDa type-I transmembrane p-glycoprotein (Marhaba & Zoller, 2004), which recognises hyaluronic acid of the extracellular matrix. CD44 has a broad range of signalling functions such as proliferation, apoptosis, survival, migration and differentiation, depending on the cell type CD44 is expressed on, the proteins interacting, as well as the CD44-epitope bound (S. J. Wang & Bourguignon, 2006). Anti-CD44 antibody represents the major anti-CSC approach. Young *et al.* reported the humanised anti-CD44 mAb (HuARH460-16-2) inhibited almost 80% tumour growth in pancreatic cancer xenografts (Young, 2007). In a melanoma xenograft study, GKW.A2 anti-CD44 mAb showed a decrease in tumour metastasis and increase in animal survival (Guo et al., 1994). In head and neck cancer, CD44 has been suggested to co-localise with EGFR and co-regulate CSC signalling (Kawano et al., 2005), which caused chemotherapy resistance (S. J. Wang & Bourguignon, 2006). This opens up the opportunities for anti-CD44/EGFR combination antibody therapy. CD24 is a mucin-like GPI-anchored protein, with multiple O- or N-linked glycosylation sites. It is involved in cell selection/ maturation in haematopoiesis and embryonic development (Kristiansen, Sammar, & Altevogt, 2004). It is found to be overexpressed in 90% of colorectal cancer (Sagiv et al., 2006). BCSCs express low levels of CD24. It has been suggested that the interaction between CD24 and P-selectin is crucial for the rolling of breast cancer cells and elevated levels are found in invasive breast cancer suggesting its role in metastasis (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). An anti-CD24 mAb inhibited colorectal and pancreatic tumour growth *in vitro* and *in vivo* and the mechanism was suggested to be mediated through CD24 degradation (Sagiv et al., 2008).

Noto and coworkers have demonstrated that CSCs could be isolated from oral squamous cell carcinoma derived cell line using CD44 and SSEA-4 as markers. They showed that CD44 and SSEA-4 double positive cells had the renewal capacity and stem cell gene expression profiles. Furthermore, these CD44 and SSEA-4 positive CSCs could initiate tumours from a minimum number of cells in

immunodeficient mice (Noto et al., 2013). Importantly, SSEA-4 was identified as a marker of adriamycin/cyclophosphamide-based (A/C) chemotherapy resistance cancer cells in 4 TNBC patient-derived xenograft (PDX) models, which responded to but failed to reach complete a pathological response. Moreover, high expression of α-2,3-sialyltransferase was significantly associated with poor outcome in ER and PgR negative breast cancer patients and ovarian cancer patients treated with chemotherapy (Aloia et al., 2015). Thus, other than breast cancer, FG2811.72 mAb could be used in targeting radiotherapy and chemotherapy resistance SSEA-4^+ BCSCs, which may be useful in preventing breast cancer recurrence and metastasis. In addition, the use of combinations of mAbs to modulate two different but related tumour signalling pathways is emerging as a successful clinical approach(Hainsworth et al., 2008; Lin et al., 2009). Therefore, the combination of FG2811.72 mAb with other mAbs against solid tumour targets and CSC targets could potentially destroy the entire tumour and the resilient CSC population, preventing tumour recurrence. The generation of bi-specific mAb recognising SSEA-4 and CSC markers could be used to increase the specificity of CSC targeting.

Immunohistochemical analysis of SSEA-4 expression on a normal human tissue microarray composed of 31 different tissues utilising FG2811.72 showed that the mAb only stained testis, liver and pancreas. Unlike FG2811.72, MC813-70 mAb stained more normal human tissues compared to FG2811.72 due to its poor specificity. MC813-70 stained testis, pancreas, placenta, bladder, brain, testis, kidney as well as macrophage like cells in the breast and cervix.

Both SSEA-3 and SSEA-4 have been reported to be expressed as a minor GSL in erythrocytes, defined by MC631 and MC813 mAbs respectively (Tippett et al., 1986). However, FG2811.72 showed no reactivity with erythrocytes from all five healthy donors. Together these results suggest that, SSEA-4 is absent from many normal tissues as well as erythrocytes. Unlike MC631 and MC813 mAbs, FG2811.72 mAb recognises SSEA-4 specifically whereas MC631, although is an anti-SSEA-3 mAb [SSEA-3; (Gal(β1-3)GalNac(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer)], cross reacts with Globo-H (GalNac(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer) and SSEA-4 (NeuAc(α2-3)Gal(β1-3)GalNac(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer); and MC813 mAb although is an anti-SSEA-4 mAb, it also cross reacts with SSEA-3, Globo-H and Forssman. Previous studies showing SSEA-4 expression in many normal human tissues other than testis, liver and pancreas as well as erythrocytes defined by MC813 mAbs therefore might be due to the cross-reactivity of the mAbs recognition of other glycoepitopes.

Carbohydrate-recognising mAbs generally exhibit weak glycan affinity. Conversely, FG2811.72 mAb displayed strong functional affinity for SSEA-4 glycolipid as well as SSEA-3/4-LMTK cells which overexpressed the SSEA-3 and SSEA-4 glycolipids. This could be due to the IgG3 isotype of the mAb.

The most common mAb isotype induced against glycans in mice is the IgG3 subtype (Abdelmoula et al., 1989; Greenspan, Monafo, & Davie, 1987). Mouse IgG3 antibodies have the ability to form non-covalent interactions between Fc regions of adjacent IgG3 molecules when bound to high density antigen (Breedveld, 2000; Grey, Hirst, & Cohn, 1971). These non-covalent interactions stabilise surface-bound antibody thereby reducing target dissociation rates. The Fc-mediated self-association could be a mechanism by which functional avidity is increased, in particular to multivalent antigens (Yoo et al., 2003).

FG2811.72 mAb induces PI uptake on SSEA-3/4-LMTK cells and inhibited SSEA-3/4-LMTK and SKOV3 cell growth supporting our previous studies in which, glycolipid antigens (G6B3R9) but not glycoprotein antigens (G6B14R9), are responsible in inducing direct tumour cell death/growth inhibition in an oncolytic manner. In addition, the internalisation of FG2811.72 mAb into cells further supports our previous studies suggesting FG88.2 mAb internalisation could be via both glycolipid and glycoprotein antigens, although the internalisation mechanisms remain unexplored. FG2811.72 mAb also induces ADCC and CDC cytotoxicity against high antigen expression cancer cells. To date, murine IgG3 MC813 mAb is the only anti-SSEA-4 mAb shown to induce CDC against glioblastoma multiforme (GBM) cell lines *in vitro* and suppressed GBM tumour growth in mice (Lou et al., 2014).

In summary, the involvement of sialylated glycoconjugates in cancer progression and metastasis makes them highly relevant as potential anti-metastatic targets. The discovery of inhibiting tumour cell growth by targeting glycolipid antigens is exciting and will undoubtedly lead to new diagnostic and therapeutic mAbs. Furthermore, additional work and investigation in this area may lead to more effective strategies for cancer prevention, diagnosis and treatment.

Chapter 7: Final discussion

To generate mAbs against TACAs was challenging due to fact that glycolipids are poorly immunogenic. Therefore to tackle the problem of antigen immunogenicity, a novel immunisation strategy was used. Mice were immunised with tumour cell plasma membrane glycolipid extracts incorporated into liposomes in the presence of anti-CD40 mAb and α-GalCer as adjuvants. Two high affinity (with dissociation constants of Nano molar range), IgG3 murine mAbs (FG88.2 and FG88.7) were generated suggesting the immunisation protocol improved the immunogenicity of the glycolipid antigens.

CD40 is one of the co-stimulatory molecules required to increase the antigen-presenting function and proliferation of resting B cells (Nomura et al., 1995; van Kooten & Banchereau, 2000). In addition, on DCs, CD40 has been found to be a critical step in the final maturation of DCs into fully competent APCs. CD40L or CD154 is the natural ligand for CD40 and is mainly expressed on activated CD4+ T cells (van Kooten & Banchereau, 2000). CD40-CD40L interaction on DCs has been shown to enhance the expression of the MHC and costimulatory molecules on the APC as well as the secretion of proinflammatory cytokines, IL-12, which facilitate and improve both naïve CD4+ and CD8+ T cell activation (Bennett et al., 1998; van Kooten & Banchereau, 2000). The anti-CD40 mAb used in this immunisation regimen was meant to mimic the function of CD40L and thus substitute or enhance the function of CD4+ helper T cells. CD4+ T helper cell signals are critical to the development of high affinity, class-switched, B cell responses.

Recently, another subset of T cells has been identified which provides help for B cell proliferation and antibody production (P. Barral et al., 2008; Leadbetter et al., 2008), known as invariant natural killer T (iNKT) cells. iNKT cells expresses an invariant TCR and NK marker such as NK1.1 (in mice) or CD161 (in humans) (Bendelac, Rivera, Park, & Roark, 1997; Kronenberg, 2005). Unlike conventional T cells, iNKT cells do not recognise peptide antigens presented by polymorphic MHCI or MHCII molecules but glycolipid antigens presented by the non polymorphic CD1d antigen presenting molecules (Bendelac et al., 1997; Brigl & Brenner, 2004, 2010; Kronenberg, 2005). α-GalCer is a glycolipid antigen known to specifically activate iNKT cells (Borg et al., 2007; Koch et al., 2005; Tsuji, 2006). After presentation of α-GalCer by CD1d molecules, α-GalCer activates iNKT cells to rapidly produce large quantities of Th1 (IFN-γ) and Th2 (IL-4) cytokines, and subsequently induces the activation of DCs, NK cells, B cells and CD4+ and CD8+ T cells (Kronenberg, 2005). α-GalCer can therefore be used as an adjuvant to enhance the maturation and proliferation of B cells to make high affinity, class switched mAbs against glycolipid antigens.

Liposomes are synthetic lipid spheres that act as both vaccine delivery vehicles and adjuvants (Mohan, Verma, & Rao, 2013). Liposomes can encapsulate water-soluble antigens (proteins, peptides, nucleic acids, carbohydrates, haptens) within the aqueous inner space of liposomes or intercalate lipophilic compounds (lipopeptides, antigens, adjuvants, linker molecules) into the lipid bilayer. (Ronnberg, Fekadu, & Morein, 1995; Schwendener, 2014; D. S. Watson, Endsley, & Huang, 2012). One of the major advantages of liposomes is the 'depot effect', in which the liposomal carrier helps to extend the half-life of the antigen and adjuvant thereby increasing the time of antigen exposure to the immune cells (Henriksen-Lacey et al., 2010).

Several criteria have been described for the selection of anti-tumour mAbs: (1) the mAb binds to cell surface tumour antigen, (2) the mAb binds to tumour antigen at high affinity, (3) the mAb recognises tumour antigen that is overexpressed on tumours but has limited expression on normal tissues, (4) the mAb has potent immune mediated and non-immune mediated cytotoxicity effects, (5) the mAb directly kills tumour cells and/or (6) the mAb internalises into target cells so it can delivery toxic payloads.

FG88 mAbs recognise $Le^{a/c/x}$ glycoepitopes at subnanomolar potency. $Le^{a/c/x}$ glycoepitopes, which can be found on both glycoprotein and glycolipid antigens, were found to be expressed on the surface of many different cell lines as defined by flow cytometry analysis. Efficacy and safety remain the main criteria for the generation of therapeutic mAbs against cancer. It is desirable that the target antigens are overexpressed on a broad range of tumour types but have low cross reactivity to normal tissues, thereby reducing toxicity in cancer patients. The FG88.2 mAb showed excellent tumour cell surface antigen binding and good levels of binding to a large percentage of tumours with low level binding to a limited number of normal tissues by immunohistochemistry. The significant association of strong FG88.2 binding with poor outcome in the colorectal sample cohort, independent of stage and vascular invasion, suggests that FG88.2 mAb has great potential targeting the most aggressive colorectal cancers. Most importantly, this study is the first to demonstrate independent prognostic value for $Le^aLe^cLe^x$ expression in colorectal cancer as defined by the FG88.2 mAb.

To generate efficient therapeutic mAbs, the effector functions of mAbs are crucial. Ideally, therapeutic mAbs should have multiple effector functions (ADCC, CDC, neutralisation effect, induction of apoptosis, and inhibition of angiogenesis), as most approved therapeutic mAbs in the clinic induce tumour cell death via more than one mechanism. The best example being Rituximab, which is an anti-CD20 mAb, has been shown to eliminate CD20+ malignant B cells via three different mechanisms; ADCC, CDC and apoptosis (Pescovitz, 2006). FG88 mAbs induce potent ADCC, CDC and direct killing of tumour cells via oncosis. It has been well accepted that tumours are able to

manipulate the tumour microenvironment by releasing cytokines and other soluble factors, which create an immunosuppressive environment (Ostman & Augsten, 2009). By releasing tumour cellular contents via mAb-induced pores, the FG88 mAbs might be able to eradicate tumours completely by recruiting immune effector cells to tumour sites. The *in vivo* study was the best evidence showing that FG88 mAbs are capable of eradicate both primary and metastatic tumours.

Although FG88 mAbs internalise efficiently into tumour cells, part of the mAbs remain binding on the tumour cell surface, which makes them attractive mAbs to be used as antibody drug conjugates (ADCs) without affecting their immune mediated as well as direct cytotoxicities.

FG88.2 mAb showed weak to moderate binding to the normal gastrointestinal tissues, but this is mainly localised to apical cells and it is unclear whether the mAb would be accessible to these cells. Although the basis for the selection of target antigen for an ADC is high expression level in tumour tissues with restricted normal tissue distribution, it is important to note that, antigen expression level in normal tissues may not necessarily always linked to subsequent toxicity in clinical trials. Several ADCs with normal tissue cross reactivity have shown evidence of effective clinical activities while causing only minimal adverse effects. Examples are cantuzimab mertansine (directed against CanAg) (Helft et al., 2004; Rodon et al., 2008; Tolcher et al., 2003) and Indatuximab Ravtansine (directed against CD138) (Romano et al., 2014) (ASH 2013, abstract number 758).

Chimerisation or humanisation of murine mAbs is important because murine mAbs would cause human anti-mouse antibody (HAMA) response. Unfortunately, our mAbs functional avidity is lost when they were converted from murine IgG3 FG88.2 mAb to chimeric IgG1. Wolff *et al.* reported the same observation when the murine BR96 mAb was chimerised (Wolff et al., 1993). In order to restore the immunological function of the CH88.2 mAb, the E345R mutation [a single amino acid substitution at position E345R ($Glu^{345}\rightarrow$ Arg)] was introduced into CH88.2 to generate a mutant version of CH88.2 named ECH88.2 mAb. The E345R mutation has been reported to be responsible for antibody hexamer formation and a general C1 activation enhancer for all IgG isotype variants (Diebolder et al., 2014). After including the E345R mutation, the direct cell killing ability of ECH88.2 mAb was restored and the CDC activity was improved.

In this study, the FG88 antigens appeared to be excellent mAb targets in cancer therapy. However, the contribution of glycoprotein or glycolipid antigens to the cytotoxic and internalisation effects of the FG88 mAbs remain unknown.

G6B3R9 expresses Le^a antigen predominantly on glycolipids whereas G6B14R9 predominantly expresses Le^a on glycoproteins. By utilising these two cell lines, our initial results suggest that FG88

glycoepitopes on glycolipids are responsible for the direct killing effect of the mAb and both glycolipid and glycoprotein antigens are involved in antigen internalisation. The internalisation pathways of FG88 mAbs remained unexplored but our next aim would be to study the internalisation mechanisms involved. In addition, it would be interesting to study the direct killing mechanism (oncosis) used by these mAbs to kill tumours. Furthermore, a toxicity test in the cynomolgus monkey model using FG88.2 mAb is planned to check for possible toxicity.

Despite advances in cancer treatment, many still fail, resulting in reduced overall survival of cancer patients. Chemotherapy, radiotherapy, surgical resection alone or in combination are the most commonly used cancer therapies. These standard cancer therapies often have only temporary effects on tumours and patients tend to relapse. Evidence has emerged that CSCs represent a subpopulation of cells within cancers that are highly resistant to chemotherapy and radiation (Massard, Deutsch, & Soria, 2006).

We produced an ultra-specific anti-SSEA-4 CSC mAb. The previous anti-SSEA-4 mAb (MC813-70) which had been reported on extensively in the literature for decades was shown to recognise not only SSEA-4, but also SSEA-3 and Forssman glycans. To our knowledge, no other SSEA-4 specific IgG mAb has been described. In addition, it can be used to further characterise the SSEA-4 glycolipid antigen, i.e. SSEA-4 expression on tumour, normal tissues and human stem cells.

FG2811.72 bound to normal human testis, liver and pancreatic tissues, as opposed to breast, colon, gastrointestinal tract, kidney, lung, ovary, pancreas, rectum, stomach, testes, thymus and uterine cervix normal tissues as reported using the non-specific MC813-70 mAb (Lou et al., 2014). MC813-70 mAb showed binding to erythrocytes and caused hemagglutination (Cooling & Hwang, 2005). In contrast, FG2811.72 did not bind to erythrocytes nor cause hemmagglutination, suggesting that SSEA-4 is absent from erythrocytes. In addition to ADCC and CDC, FG2811.72 mAb also induces PI uptake on SSEA-3/4-LMTK cells and inhibited SSEA-3/4-LMTK cell growth, indicating that the SSEA-4 glycolipid has great potential as a tumour target. To our knowledge, there is no pure glycolipid target that has been shown to internalise into tumour cells upon mAb binding. In this study, preliminary results showFG2811.72 is internalised into tumour cells, suggesting SSEA-4 and other glycolipid antigens may have great potential as ADC targets.

In conclusion, our findings suggest that high affinity anti-glycan and anti-glycolipid mAbs can be generated and they can bind to tumour antigens with high specificity and induce potent cytotoxicity towards tumours with the help of immune effector cells, complement proteins and/or directly. By introducing the E345R mutation, the antigen binding affinity, direct killing ability and CDC activity of

our chimeric mAbs can be regained. Most importantly, although the production of anti-glycolipid mAbs against cancers is not new, the production of ultraspecific high affinity IgG mAbs against glycolipid antigens is novel. FG88 and FG2811.72 mAbs definitely have great potential in cancer therapy and serve as valuable tools in glycobiology research.

Bibliography

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2007). Cellular and molecular immunology (6th ed.). Philadelphia: Saunders Elsevier.

Abdel-Fatah, T., Arora, A., Gorguc, I., Abbotts, R., Beebeejaun, S., Storr, S., . . . Madhusudan, S. (2013). Are DNA repair factors promising biomarkers for personalized therapy in gastric cancer? Antioxid Redox Signal, 18(18), 2392-2398. doi: 10.1089/ars.2012.4873

Abdelmoula, M., Spertini, F., Shibata, T., Gyotoku, Y., Luzuy, S., Lambert, P. H., & Izui, S. (1989). IgG3 is the major source of cryoglobulins in mice. J Immunol, 143(2), 526-532.

Agadjanyan, M., Luo, P., Westerink, M. A., Carey, L. A., Hutchins, W., Steplewski, Z., . . . Kieber-Emmons, T. (1997). Peptide mimicry of carbohydrate epitopes on human immunodeficiency virus. [Research Support, U.S. Gov't, Non-P.H.S.

Research Support, U.S. Gov't, P.H.S.]. Nat Biotechnol, 15(6), 547-551. doi: 10.1038/nbt0697-547

Agematsu, K., Nagumo, H., Shinozaki, K., Hokibara, S., Yasui, K., Terada, K., . . . Komiyama, A. (1998). Absence of IgD-CD27(+) memory B cell population in X-linked hyper-IgM syndrome. J Clin Invest, 102(4), 853-860. doi: 10.1172/jci3409

Akira, S., Okazaki, K., & Sakano, H. (1987). Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science, 238(4830), 1134-1138.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A, 100(7), 3983-3988. doi: 10.1073/pnas.0530291100

Alduaij, W., Ivanov, A., Honeychurch, J., Cheadle, E. J., Potluri, S., Lim, S. H., . . . Illidge, T. M. (2011). Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell malignancies. Blood, 117(17), 4519-4529. doi: 10.1182/blood-2010-07-296913

Allen, C. D., Okada, T., & Cyster, J. G. (2007). Germinal-center organization and cellular dynamics. Immunity, 27(2), 190-202. doi: 10.1016/j.immuni.2007.07.009

Alley, W. R., Jr., & Novotny, M. V. (2010). Glycomic analysis of sialic acid linkages in glycans derived from blood serum glycoproteins. J Proteome Res, 9(6), 3062-3072. doi: 10.1021/pr901210r

Aloia, A., Petrova, E., Tomiuk, S., Bissels, U., Deas, O., Saini, M., . . . Hardt, O. (2015). The sialyl-glycolipid stage-specific embryonic antigen 4 marks a subpopulation of chemotherapy-resistant breast cancer cells with mesenchymal features. Breast Cancer Res, 17(1), 146. doi: 10.1186/s13058-015-0652-6

Alt, F. W., Yancopoulos, G. D., Blackwell, T. K., Wood, C., Thomas, E., Boss, M., . . . Baltimore, D. (1984). Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J, 3(6), 1209-1219.

Altevogt, P., Fogel, M., Cheingsong-Popov, R., Dennis, J., Robinson, P., & Schirrmacher, V. (1983). Different patterns of lectin binding and cell surface sialylation detected on related high- and low-metastatic tumor lines. Cancer Res, 43(11), 5138-5144.

Amanna, I. J., Carlson, N. E., & Slifka, M. K. (2007). Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med, 357(19), 1903-1915. doi: 10.1056/NEJMoa066092

Amu, S., Saunders, S. P., Kronenberg, M., Mangan, N. E., Atzberger, A., & Fallon, P. G. (2010). Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. J Allergy Clin Immunol, 125(5), 1114-1124 e1118. doi: 10.1016/j.jaci.2010.01.018

Anderson, A. C., Reddy, J., Nazareno, R., Sobel, R. A., Nicholson, L. B., & Kuchroo, V. K. (2004). IL-10 plays an important role in the homeostatic regulation of the autoreactive repertoire in naive mice. J Immunol, 173(2), 828-834.

Anderson, R. G., & Jacobson, K. (2002). A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. Science, 296(5574), 1821-1825. doi: 10.1126/science.1068886

Andre, F., & Zielinski, C. C. (2012). Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. Ann Oncol, 23 Suppl 6, vi46-51. doi: 10.1093/annonc/mds195

Andrews, P. W. (1998). Teratocarcinomas and human embryology: pluripotent human EC cell lines. Review article. [Research Support, Non-U.S. Gov't

Review]. APMIS, 106(1), 158-167; discussion 167-158.

Andrews, P. W. (2011). Toward safer regenerative medicine. Nat Biotechnol, 29(9), 803-805. doi: 10.1038/nbt.1974

Angata, T., & Varki, A. (2002). Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. Chem Rev, 102(2), 439-469.

Angstrom, J., Teneberg, S., & Karlsson, K. A. (1994). Delineation and comparison of ganglioside-binding epitopes for the toxins of Vibrio cholerae, Escherichia coli, and Clostridium tetani: evidence for overlapping epitopes. Proc Natl Acad Sci U S A, 91(25), 11859-11863.

Apweiler, R., Hermjakob, H., & Sharon, N. (1999). On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim Biophys Acta, 1473(1), 4-8.

Ashkenazi, A., & Dixit, V. M. (1998). Death receptors: signaling and modulation. Science, 281(5381), 1305-1308.

Atkinson, B. F., Ernst, C. S., Herlyn, M., Steplewski, Z., Sears, H. F., & Koprowski, H. (1982). Gastrointestinal cancer-associated antigen in immunoperoxidase assay. [Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. Cancer Res, 42(11), 4820-4823.

Awate, S., Babiuk, L. A., & Mutwiri, G. (2013). Mechanisms of action of adjuvants. Front Immunol, 4, 114. doi: 10.3389/fimmu.2013.00114

Balk, S. P., Bleicher, P. A., & Terhorst, C. (1991). Isolation and expression of cDNA encoding the murine homologues of CD1. J Immunol, 146(2), 768-774.

Ballare, C., Barrio, M., Portela, P., & Mordoh, J. (1995). Functional properties of FC-2.15, a monoclonal antibody that mediates human complement cytotoxicity against breast cancer cells. Cancer Immunol Immunother, 41(1), 15-22.

Ballare, C., Portela, P., Schiaffi, J., Yomha, R., & Mordoh, J. (1998). Reactivity of monoclonal antibody FC-2.15 against drug resistant breast cancer cells. Additive cytotoxicity of adriamycin and taxol with FC-2.15. Breast Cancer Res Treat, 47(2), 163-170.

Banin Hirata, B. K., Oda, J. M., Losi Guembarovski, R., Ariza, C. B., de Oliveira, C. E., & Watanabe, M. A. (2014). Molecular markers for breast cancer: prediction on tumor behavior. [Review]. Dis Markers, 2014, 513158. doi: 10.1155/2014/513158

Banks, N. D., Kinsey, N., Clements, J., & Hildreth, J. E. (2002). Sustained antibody-dependent cell-mediated cytotoxicity (ADCC) in SIV-infected macaques correlates with delayed progression to AIDS. AIDS Res Hum Retroviruses, 18(16), 1197-1205. doi: 10.1089/08892220260387940

Barnes, D. M. (1993). c-erbB-2 amplification in mammary carcinoma. [Review]. J Cell Biochem Suppl, 17G, 132-138.

Barnham, K. J., Torres, A. M., Alewood, D., Alewood, P. F., Domagala, T., Nice, E. C., & Norton, R. S. (1998). Role of the 6-20 disulfide bridge in the structure and activity of epidermal growth factor. Protein Sci, 7(8), 1738-1749. doi: 10.1002/pro.5560070808

Barral, D. C., & Brenner, M. B. (2007). CD1 antigen presentation: how it works. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't

Review]. Nat Rev Immunol, 7(12), 929-941. doi: 10.1038/nri2191

Barral, P., Eckl-Dorna, J., Harwood, N. E., De Santo, C., Salio, M., Illarionov, P., . . . Batista, F. D. (2008). B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. [Research Support, Non-U.S. Gov't]. Proc Natl Acad Sci U S A, 105(24), 8345-8350. doi: 10.1073/pnas.0802968105

Bassing, C. H., Swat, W., & Alt, F. W. (2002). The mechanism and regulation of chromosomal V(D)J recombination. Cell, 109 Suppl, S45-55.

Bastiani, M., & Parton, R. G. (2010). Caveolae at a glance. J Cell Sci, 123(Pt 22), 3831-3836. doi: 10.1242/jcs.070102

Beattie, E. C., Howe, C. L., Wilde, A., Brodsky, F. M., & Mobley, W. C. (2000). NGF signals through TrkA to increase clathrin at the plasma membrane and enhance clathrin-mediated membrane trafficking. J Neurosci, 20(19), 7325-7333.

Beckman, E. M., Melian, A., Behar, S. M., Sieling, P. A., Chatterjee, D., Furlong, S. T., . . . Porcelli, S. A. (1996). CD1c restricts responses of mycobacteria-specific T cells. Evidence for antigen presentation by a second member of the human CD1 family. J Immunol, 157(7), 2795-2803.

Beckman, E. M., Porcelli, S. A., Morita, C. T., Behar, S. M., Furlong, S. T., & Brenner, M. B. (1994). Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. Nature, 372(6507), 691-694. doi: 10.1038/372691a0

Bektas, M., & Spiegel, S. (2004). Glycosphingolipids and cell death. Glycoconj J, 20(1), 39-47. doi: 10.1023/B:GLYC.0000016741.88476.8b

Bendelac, A., Rivera, M. N., Park, S. H., & Roark, J. H. (1997). Mouse CD1-specific NK1 T cells: development, specificity, and function. [Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.

Review]. Annu Rev Immunol, 15, 535-562. doi: 10.1146/annurev.immunol.15.1.535

Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., & Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. [Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. Nature, 393(6684), 478-480. doi: 10.1038/30996

Berek, C., Berger, A., & Apel, M. (1991). Maturation of the immune response in germinal centers. Cell, 67(6), 1121-1129.

Berger, A. C., Meszoely, I. M., Ross, E. A., Watson, J. C., & Hoffman, J. P. (2004). Undetectable preoperative levels of serum CA 19-9 correlate with improved survival for patients with resectable pancreatic adenocarcinoma. Ann Surg Oncol, 11(7), 644-649. doi: 10.1245/ASO.2004.11.025

Berland, R., & Wortis, H. H. (2002). Origins and functions of B-1 cells with notes on the role of CD5. Annu Rev Immunol, 20, 253-300. doi: 10.1146/annurev.immunol.20.100301.064833

Berlin, J. D., Catalano, P., Thomas, J. P., Kugler, J. W., Haller, D. G., & Benson, A. B., 3rd. (2002). Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. J Clin Oncol, 20(15), 3270-3275.

Birkle, S., Zeng, G., Gao, L., Yu, R. K., & Aubry, J. (2003). Role of tumor-associated gangliosides in cancer progression. [Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.

Review]. Biochimie, 85(3-4), 455-463.

Blaszczyk, M., Pak, K. Y., Herlyn, M., Sears, H. F., & Steplewski, Z. (1985). Characterization of Lewis antigens in normal colon and gastrointestinal adenocarcinomas. Proc Natl Acad Sci U S A, 82(11), 3552-3556.

Bogdanovic, E., Coombs, N., & Dumont, D. J. (2009). Oligomerized Tie2 localizes to clathrin-coated pits in response to angiopoietin-1. Histochem Cell Biol, 132(2), 225-237. doi: 10.1007/s00418-009-0603-3

Bonneterre, J., Thurlimann, B., Robertson, J. F., Krzakowski, M., Mauriac, L., Koralewski, P., . . . von Euler, M. (2000). Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex Randomized Group Efficacy and Tolerability study. [Clinical Trial

Multicenter Study

Randomized Controlled Trial

Research Support, Non-U.S. Gov't]. J Clin Oncol, 18(22), 3748-3757.

Borg, N. A., Wun, K. S., Kjer-Nielsen, L., Wilce, M. C., Pellicci, D. G., Koh, R., . . . Rossjohn, J. (2007). CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't]. Nature, 448(7149), 44-49. doi: 10.1038/nature05907

Bouchard, H., Viskov, C., & Garcia-Echeverria, C. (2014). Antibody-drug conjugates-a new wave of cancer drugs. Bioorg Med Chem Lett, 24(23), 5357-5363. doi: 10.1016/j.bmcl.2014.10.021

Boulton, S. J., & Jackson, S. P. (1996). Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. EMBO J, 15(18), 5093-5103.

Bourgeois, C., Rocha, B., & Tanchot, C. (2002). A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. [Research Support, Non-U.S. Gov't]. Science, 297(5589), 2060-2063. doi: 10.1126/science.1072615

Brahmer, J. R., Tykodi, S. S., Chow, L. Q., Hwu, W. J., Topalian, S. L., Hwu, P., . . . Wigginton, J. M. (2012). Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med, 366(26), 2455-2465. doi: 10.1056/NEJMoa1200694

Breedveld, F. C. (2000). Therapeutic monoclonal antibodies. Lancet, 355(9205), 735-740.

Brekke, O. H., Bremnes, B., Sandin, R., Aase, A., Michaelsen, T. E., & Sandlie, I. (1993). Human IgG3 can adopt the disulfide bond pattern characteristic for IgG1 without resembling it in complement mediated cell lysis. Mol Immunol, 30(16), 1419-1425.

Bretscher, P., & Cohn, M. (1970). A theory of self-nonself discrimination. Science, 169(3950), 1042-1049.

Bretscher, P. A. (1999). A two-step, two-signal model for the primary activation of precursor helper T cells. Proc Natl Acad Sci U S A, 96(1), 185-190.

Brigl, M., & Brenner, M. B. (2004). CD1: antigen presentation and T cell function. [Review]. Annu Rev Immunol, 22, 817-890. doi: 10.1146/annurev.immunol.22.012703.104608

Brigl, M., & Brenner, M. B. (2010). How invariant natural killer T cells respond to infection by recognizing microbial or endogenous lipid antigens. [Research Support, N.I.H., Extramural

Review]. Semin Immunol, 22(2), 79-86. doi: 10.1016/j.smim.2009.10.006

Brockhausen, I. (2006). Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. EMBO Rep, 7(6), 599-604. doi: 10.1038/sj.embor.7400705

Brooks, S. A., & Leathem, A. J. (1995). Expression of the CD15 antigen (Lewis x) in breast cancer. Histochem J, 27(9), 689-693.

Brossay, L., Chioda, M., Burdin, N., Koezuka, Y., Casorati, G., Dellabona, P., & Kronenberg, M. (1998). CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. J Exp Med, 188(8), 1521-1528.

Bruggemann, M., Williams, G. T., Bindon, C. I., Clark, M. R., Walker, M. R., Jefferis, R., . . . Neuberger, M. S. (1987). Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. J Exp Med, 166(5), 1351-1361.

Bruhns, P. (2012). Properties of mouse and human IgG receptors and their contribution to disease models. Blood, 119(24), 5640-5649. doi: 10.1182/blood-2012-01-380121

Bruhns, P., Iannascoli, B., England, P., Mancardi, D. A., Fernandez, N., Jorieux, S., & Daeron, M. (2009). Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood, 113(16), 3716-3725. doi: 10.1182/blood-2008-09-179754

Bull, C., Stoel, M. A., den Brok, M. H., & Adema, G. J. (2014). Sialic acids sweeten a tumor's life. Cancer Res, 74(12), 3199-3204. doi: 10.1158/0008-5472.can-14-0728

Busslinger, M. (2004). Transcriptional control of early B cell development. Annu Rev Immunol, 22, 55-79. doi: 10.1146/annurev.immunol.22.012703.104807

Cahan, L. D., Irie, R. F., Singh, R., Cassidenti, A., & Paulson, J. C. (1982). Identification of a human neuroectodermal tumor antigen (OFA-I-2) as ganglioside GD2. Proc Natl Acad Sci U S A, 79(24), 7629-7633.

Calabi, F., Jarvis, J. M., Martin, L., & Milstein, C. (1989). Two classes of CD1 genes. Eur J Immunol, 19(2), 285-292. doi: 10.1002/eji.1830190211

Callahan, M. K., & Wolchok, J. D. (2013). At the bedside: CTLA-4- and PD-1-blocking antibodies in cancer immunotherapy. [Research Support, N.I.H., Extramural

Review]. J Leukoc Biol, 94(1), 41-53. doi: 10.1189/jlb.1212631

Cambier, J. C. (1995). Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). J Immunol, 155(7), 3281-3285.

Camp, R. L., Dolled-Filhart, M., & Rimm, D. L. (2004). X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Clin Cancer Res, 10(21), 7252-7259. doi: 10.1158/1078-0432.ccr-04-0713

Campbell, A. K., & Morgan, B. P. (1985). Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack. Nature, 317(6033), 164-166.

Canfield, S. M., & Morrison, S. L. (1991). The binding affinity of human IgG for its high affinity Fc receptor is determined by multiple amino acids in the CH2 domain and is modulated by the hinge region. J Exp Med, 173(6), 1483-1491.

Capelan, M., Pugliano, L., De Azambuja, E., Bozovic, I., Saini, K. S., Sotiriou, C., . . . Piccart-Gebhart, M. J. (2013). Pertuzumab: new hope for patients with HER2-positive breast cancer. [Review]. Ann Oncol, 24(2), 273-282. doi: 10.1093/annonc/mds328

Capurro, M., Ballare, C., Bover, L., Portela, P., & Mordoh, J. (1999). Differential lytic and agglutinating activity of the anti-Lewis(X) monoclonal antibody FC-2.15 on human polymorphonuclear neutrophils and MCF-7 breast tumor cells. In vitro and ex vivo studies. Cancer Immunology Immunotherapy, 48(2-3), 100-108. doi: DOI 10.1007/s002620050553

Carey, L. A. (2010). Directed therapy of subtypes of triple-negative breast cancer. Oncologist, 15 Suppl 5, 49-56. doi: 10.1634/theoncologist.2010-S5-49

Carpentier, J. L., Gorden, P., Anderson, R. G., Goldstein, J. L., Brown, M. S., Cohen, S., & Orci, L. (1982). Co-localization of 125I-epidermal growth factor and ferritin-low density lipoprotein in coated pits: a quantitative electron microscopic study in normal and mutant human fibroblasts. J Cell Biol, 95(1), 73-77.

Carter, P. J., & Senter, P. D. (2008). Antibody-drug conjugates for cancer therapy. Cancer J, 14(3), 154-169. doi: 10.1097/PPO.0b013e318172d704

Cary, R. B., Peterson, S. R., Wang, J., Bear, D. G., Bradbury, E. M., & Chen, D. J. (1997). DNA looping by Ku and the DNA-dependent protein kinase. Proc Natl Acad Sci U S A, 94(9), 4267-4272.

Casi, G., & Neri, D. (2012). Antibody-drug conjugates: basic concepts, examples and future perspectives. J Control Release, 161(2), 422-428. doi: 10.1016/j.jconrel.2012.01.026

Cazet, A., Julien, S., Bobowski, M., Burchell, J., & Delannoy, P. (2010). Tumour-associated carbohydrate antigens in breast cancer. Breast Cancer Res, 12(3), 204. doi: 10.1186/bcr2577

Chames, P., Van Regenmortel, M., Weiss, E., & Baty, D. (2009). Therapeutic antibodies: successes, limitations and hopes for the future. Br J Pharmacol, 157(2), 220-233. doi: 10.1111/j.1476-5381.2009.00190.x

Chandrasekaran, E. V., Davila, M., Nixon, D. W., Goldfarb, M., & Mendicino, J. (1983). Isolation and structures of the oligosaccharide units of carcinoembryonic antigen. [Research Support, U.S. Gov't, P.H.S.]. J Biol Chem, 258(11), 7213-7222.

Chang, W. W., Lee, C. H., Lee, P., Lin, J., Hsu, C. W., Hung, J. T., . . . Yu, A. L. (2008). Expression of Globo H and SSEA3 in breast cancer stem cells and the involvement of fucosyl transferases 1 and 2 in Globo H synthesis. Proc Natl Acad Sci U S A, 105(33), 11667-11672. doi: 10.1073/pnas.0804979105

Chari, R. V., Miller, M. L., & Widdison, W. C. (2014). Antibody-drug conjugates: an emerging concept in cancer therapy. Angew Chem Int Ed Engl, 53(15), 3796-3827. doi: 10.1002/anie.201307628

Chen, C., Prak, E. L., & Weigert, M. (1997). Editing disease-associated autoantibodies. Immunity, 6(1), 97-105.

Chester, M. A. (1998). IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature of glycolipids--recommendations 1997. Eur J Biochem, 257(2), 293-298.

Christiansen, D., Vaughan, H. A., Milland, J., Dodge, N., Mouhtouris, E., Smyth, M. J., . . . Sandrin, M. S. (2011). Antibody responses to glycolipid-borne carbohydrates require CD4+ T cells but not CD1 or NKT cells. Immunol Cell Biol, 89(4), 502-510. doi: 10.1038/icb.2010.166

Christiansen, M. N., Chik, J., Lee, L., Anugraham, M., Abrahams, J. L., & Packer, N. H. (2014). Cell surface protein glycosylation in cancer. Proteomics, 14(4-5), 525-546. doi: 10.1002/pmic.201300387

Chua, J. X., Vankemmelbeke, M., McIntosh, R. S., Clarke, P. A., Moss, R., Parsons, T., . . . Durrant, L. G. (2015). Monoclonal Antibodies Targeting LecLex-Related Glycans with Potent Antitumor Activity. Clin Cancer Res, 21(13), 2963-2974. doi: 10.1158/1078-0432.ccr-14-3030

Chung, Y., Chang, W. S., Kim, S., & Kang, C. Y. (2004). NKT cell ligand alpha-galactosylceramide blocks the induction of oral tolerance by triggering dendritic cell maturation. Eur J Immunol, 34(9), 2471-2479. doi: 10.1002/eji.200425027

Cid, E., Yamamoto, M., Buschbeck, M., & Yamamoto, F. (2013). Murine cell glycolipids customization by modular expression of glycosyltransferases. PLoS One, 8(6), e64728. doi: 10.1371/journal.pone.0064728

Cidon, E. U., Ellis, S. G., Inam, Y., Adeleke, S., Zarif, S., & Geldart, T. (2013). Molecular targeted agents for gastric cancer: a step forward towards personalized therapy. Cancers (Basel), 5(1), 64-91. doi: 10.3390/cancers5010064

Colucci, G., Giuliani, F., Gebbia, V., Biglietto, M., Rabitti, P., Uomo, G., . . . Lopez, M. (2002). Gemcitabine alone or with cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic cancer: a prospective, randomised phase III study of the Gruppo Oncologico dell'Italia Meridionale. American Cancer Society 94(4), 902-910.

Colucci, G., Labianca, R., Di Costanzo, F., Gebbia, V., Carteni, G., Massidda, B., . . . Perrone, F. (2010). Randomized phase III trial of gemcitabine plus cisplatin compared with single-agent gemcitabine as first-line treatment of patients with advanced pancreatic cancer: the GIP-1 study. J Clin Oncol, 28(10), 1645-1651. doi: 10.1200/jco.2009.25.4433

Conroy, T., Desseigne, F., Ychou, M., Bouche, O., Guimbaud, R., Becouarn, Y., . . . Ducreux, M. (2011). FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med, 364(19), 1817-1825. doi: 10.1056/NEJMoa1011923

Cooling, L., & Hwang, D. (2005). Monoclonal antibody B2, a marker of neuroendocrine sympathoadrenal precursors, recognizes the Luke (LKE) antigen. [Research Support, Non-U.S. Gov't]. Transfusion, 45(5), 709-716. doi: 10.1111/j.1537-2995.2005.04338.x

Copland, M. J., Baird, M. A., Rades, T., McKenzie, J. L., Becker, B., Reck, F., . . . Davies, N. M. (2003). Liposomal delivery of antigen to human dendritic cells. Vaccine, 21(9-10), 883-890.

Cox, J. C., & Coulter, A. R. (1997). Adjuvants--a classification and review of their modes of action. Vaccine, 15(3), 248-256.

Coyle, A. J., & Gutierrez-Ramos, J. C. (2001). The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. Nat Immunol, 2(3), 203-209. doi: 10.1038/85251

Croce, M. V., Isla-Larrain, M., Rabassa, M. E., Demichelis, S., Colussi, A. G., Crespo, M., . . . Segal-Eiras, A. (2007). Lewis x is highly expressed in normal tissues: a comparative immunohistochemical study and literature revision. Pathol Oncol Res, 13(2), 130-138. doi: Paor.2007.13.2.0130

Crotty, S., Felgner, P., Davies, H., Glidewell, J., Villarreal, L., & Ahmed, R. (2003). Cutting edge: long-term B cell memory in humans after smallpox vaccination. J Immunol, 171(10), 4969-4973.

Cunningham, D., Chau, I., Stocken, D. D., Valle, J. W., Smith, D., Steward, W., . . . Neoptolemos, J. P. (2009). Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J Clin Oncol, 27(33), 5513-5518. doi: 10.1200/jco.2009.24.2446

Cunningham, D., Starling, N., Rao, S., Iveson, T., Nicolson, M., Coxon, F., . . . Norman, A. R. (2008). Capecitabine and oxaliplatin for advanced esophagogastric cancer. N Engl J Med, 358(1), 36-46. doi: 10.1056/NEJMoa073149

D'Adamo, P. J., & Kelly, G. S. (2001). Metabolic and immunologic consequences of ABH secretor and Lewis subtype status. Altern Med Rev, 6(4), 390-405.

Daeron, M., Jaeger, S., Du Pasquier, L., & Vivier, E. (2008). Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev, 224, 11-43. doi: 10.1111/j.1600-065X.2008.00666.x

Dall'Acqua, W. F., Cook, K. E., Damschroder, M. M., Woods, R. M., & Wu, H. (2006). Modulation of the effector functions of a human IgG1 through engineering of its hinge region. J Immunol, 177(2), 1129-1138.

Dall'Olio, F., & Chiricolo, M. (2001). Sialyltransferases in cancer. Glycoconj J, 18(11-12), 841-850.

Dalziel, M., Crispin, M., Scanlan, C. N., Zitzmann, N., & Dwek, R. A. (2014). Emerging principles for the therapeutic exploitation of glycosylation. Science, 343(6166), 1235681. doi: 10.1126/science.1235681

Daniotti, J. L., Vilcaes, A. A., Torres Demichelis, V., Ruggiero, F. M., & Rodriguez-Walker, M. (2013). Glycosylation of glycolipids in cancer: basis for development of novel therapeutic approaches. Front Oncol, 3, 306. doi: 10.3389/fonc.2013.00306

Davies, A. M., Jefferis, R., & Sutton, B. J. (2014). Crystal structure of deglycosylated human IgG4-Fc. Mol Immunol, 62(1), 46-53. doi: 10.1016/j.molimm.2014.05.015

Davies, R. L., Grosse, V. A., Kucherlapati, R., & Bothwell, M. (1980). Genetic analysis of epidermal growth factor action: assignment of human epidermal growth factor receptor gene to chromosome 7. Proc Natl Acad Sci U S A, 77(7), 4188-4192.

Davitz, M. A., Low, M. G., & Nussenzweig, V. (1986). Release of decay-accelerating factor (DAF) from the cell membrane by phosphatidylinositol-specific phospholipase C (PIPLC). Selective modification of a complement regulatory protein. J Exp Med, 163(5), 1150-1161.

De Becker, G., Moulin, V., Pajak, B., Bruck, C., Francotte, M., Thiriart, C., . . . Moser, M. (2000). The adjuvant monophosphoryl lipid A increases the function of antigen-presenting cells. Int Immunol, 12(6), 807-815.

de Beer, T., Vliegenthart, J. F., Loffler, A., & Hofsteenge, J. (1995). The hexopyranosyl residue that is C-glycosidically linked to the side chain of tryptophan-7 in human RNase Us is alpha-mannopyranose. Biochemistry, 34(37), 11785-11789.

de la Salle, H., Mariotti, S., Angenieux, C., Gilleron, M., Garcia-Alles, L. F., Malm, D., . . . De Libero, G. (2005). Assistance of microbial glycolipid antigen processing by CD1e. Science, 310(5752), 1321-1324. doi: 10.1126/science.1115301

De Libero, G., & Mori, L. (2006). How T lymphocytes recognize lipid antigens. Febs Letters, 580(23), 5580-5587. doi: 10.1016/j.febslet.2006.08.029

De Smedt, T., Pajak, B., Muraille, E., Lespagnard, L., Heinen, E., De Baetselier, P., . . . Moser, M. (1996). Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. J Exp Med, 184(4), 1413-1424.

Dellabona, P., Padovan, E., Casorati, G., Brockhaus, M., & Lanzavecchia, A. (1994). An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. J Exp Med, 180(3), 1171-1176.

Deng, S. J., MacKenzie, C. R., Sadowska, J., Michniewicz, J., Young, N. M., Bundle, D. R., & Narang, S. A. (1994). Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display. [Comparative Study]. J Biol Chem, 269(13), 9533-9538.

Dennis, J. W., Laferte, S., Waghorne, C., Breitman, M. L., & Kerbel, R. S. (1987). Beta 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis. Science, 236(4801), 582-585.

Deonarain, M. P., Kousparou, C. A., & Epenetos, A. A. (2009). Antibodies targeting cancer stem cells: a new paradigm in immunotherapy? MAbs, 1(1), 12-25.

Derer, S., Beurskens, F. J., Rosner, T., Peipp, M., & Valerius, T. (2014). Complement in antibody-based tumor therapy. [Research Support, Non-U.S. Gov't

Review]. Crit Rev Immunol, 34(3), 199-214.

Di Costanzo, F., Carlini, P., Doni, L., Massidda, B., Mattioli, R., Iop, A., . . . Gasperoni, S. (2005). Gemcitabine with or without continuous infusion 5-FU in advanced pancreatic cancer: a randomised phase II trial of the Italian oncology group for clinical research (GOIRC). Br J Cancer, 93(2), 185-189. doi: 10.1038/sj.bjc.6602640

Dickerson, S. K., Market, E., Besmer, E., & Papavasiliou, F. N. (2003). AID mediates hypermutation by deaminating single stranded DNA. J Exp Med, 197(10), 1291-1296. doi: 10.1084/jem.20030481

Diebolder, C. A., Beurskens, F. J., de Jong, R. N., Koning, R. I., Strumane, K., Lindorfer, M. A., . . . Parren, P. W. (2014). Complement is activated by IgG hexamers assembled at the cell surface. Science, 343(6176), 1260-1263. doi: 10.1126/science.1248943

Diehl, L., den Boer, A. T., Schoenberger, S. P., van der Voort, E. I., Schumacher, T. N., Melief, C. J., . . . Toes, R. E. (1999). CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. [Research Support, Non-U.S. Gov't]. Nat Med, 5(7), 774-779. doi: 10.1038/10495

Dimitroff, C. J., Pera, P., Dall'Olio, F., Matta, K. L., Chandrasekaran, E. V., Lau, J. T., & Bernacki, R. J. (1999). Cell surface n-acetylneuraminic acid alpha2,3-galactoside-dependent intercellular adhesion of human colon cancer cells. [Research Support, U.S. Gov't, P.H.S.]. Biochem Biophys Res Commun, 256(3), 631-636. doi: 10.1006/bbrc.1999.0388

Distler, M., Pilarsky, E., Kersting, S., & Grutzmann, R. (2013). Preoperative CEA and CA 19-9 are prognostic markers for survival after curative resection for ductal adenocarcinoma of the pancreas - a retrospective tumor marker prognostic study. Int J Surg, 11(10), 1067-1072. doi: 10.1016/j.ijsu.2013.10.005

Doherty, G. J., & McMahon, H. T. (2009). Mechanisms of endocytosis. Annu Rev Biochem, 78, 857-902. doi: 10.1146/annurev.biochem.78.081307.110540

Dong, H., Strome, S. E., Salomao, D. R., Tamura, H., Hirano, F., Flies, D. B., . . . Chen, L. (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. [Research Support, U.S. Gov't, P.H.S.]. Nat Med, 8(8), 793-800. doi: 10.1038/nm730

Dorshkind, K. (2002). Multilineage development from adult bone marrow cells. Nat Immunol, 3(4), 311-313. doi: 10.1038/ni0402-311

Douillard, J. Y., Siena, S., Cassidy, J., Tabernero, J., Burkes, R., Barugel, M., . . . Gansert, J. (2010). Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. J Clin Oncol, 28(31), 4697-4705. doi: 10.1200/jco.2009.27.4860

Draper, J. S., Pigott, C., Thomson, J. A., & Andrews, P. W. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. [Research Support, Non-U.S. Gov't]. J Anat, 200(Pt 3), 249-258.

Dube, D. H., & Bertozzi, C. R. (2005). Glycans in cancer and inflammation--potential for therapeutics and diagnostics. Nat Rev Drug Discov, 4(6), 477-488. doi: 10.1038/nrd1751

Dudley, D. D., Chaudhuri, J., Bassing, C. H., & Alt, F. W. (2005). Mechanism and control of V(D)J recombination versus class switch recombination: similarities and differences. Adv Immunol, 86, 43-112. doi: 10.1016/s0065-2776(04)86002-4

Dullforce, P., Sutton, D. C., & Heath, A. W. (1998). Enhancement of T cell-independent immune responses in vivo by CD40 antibodies. [Research Support, Non-U.S. Gov't]. Nat Med, 4(1), 88-91.

Duncan, T. J., Rolland, P., Deen, S., Scott, I. V., Liu, D. T., Spendlove, I., & Durrant, L. G. (2007). Loss of IFN gamma receptor is an independent prognostic factor in ovarian cancer. [Randomized Controlled Trial]. Clin Cancer Res, 13(14), 4139-4145. doi: 10.1158/1078-0432.CCR-06-2833

Duraker, N., Hot, S., Polat, Y., Hobek, A., Gencler, N., & Urhan, N. (2007). CEA, CA 19-9, and CA 125 in the differential diagnosis of benign and malignant pancreatic diseases with or without jaundice. J Surg Oncol, 95(2), 142-147. doi: 10.1002/jso.20604

Durrant, L. G., Harding, S. J., Green, N. H., Buckberry, L. D., & Parsons, T. (2006). A new anticancer glycolipid monoclonal antibody, SC104, which directly induces tumor cell apoptosis. Cancer Res, 66(11), 5901-5909. doi: 10.1158/0008-5472.can-05-3812

Durrant, L. G., Noble, P., & Spendlove, I. (2012). Immunology in the clinic review series; focus on cancer: glycolipids as targets for tumour immunotherapy. Clin Exp Immunol, 167(2), 206-215. doi: 10.1111/j.1365-2249.2011.04516.x

Egan, M. L., Coligan, J. E., Pritchard, D. G., Schnute, W. C., Jr., & Todd, C. W. (1976). Physical characterization and structural studies of the carcinoembryonic antigen. [Research Support, U.S. Gov't, P.H.S.]. Cancer Res, 36(9 PT 2), 3482-3485.

Eldridge, J. H., Staas, J. K., Meulbroek, J. A., McGhee, J. R., Tice, T. R., & Gilley, R. M. (1991). Biodegradable microspheres as a vaccine delivery system. Mol Immunol, 28(3), 287-294.

Elola, M. T., Capurro, M. I., Barrio, M. M., Coombs, P. J., Taylor, M. E., Drickamer, K., & Mordoh, J. (2007). Lewis x antigen mediates adhesion of human breast carcinoma cells to activated endothelium. Possible involvement of the endothelial scavenger receptor C-type lectin. Breast Cancer Res Treat, 101(2), 161-174. doi: 10.1007/s10549-006-9286-9

Erickson, H. K., Widdison, W. C., Mayo, M. F., Whiteman, K., Audette, C., Wilhelm, S. D., & Singh, R. (2010). Tumor delivery and in vivo processing of disulfide-linked and thioether-linked antibody-maytansinoid conjugates. Bioconjug Chem, 21(1), 84-92. doi: 10.1021/bc900315y

Espeli, M., Mancini, S. J., Breton, C., Poirier, F., & Schiff, C. (2009). Impaired B-cell development at the pre-BII-cell stage in galectin-1-deficient mice due to inefficient pre-BII/stromal cell interactions. Blood, 113(23), 5878-5886. doi: 10.1182/blood-2009-01-198465

Espeli, M., Rossi, B., Mancini, S. J., Roche, P., Gauthier, L., & Schiff, C. (2006). Initiation of pre-B cell receptor signaling: common and distinctive features in human and mouse. Semin Immunol, 18(1), 56-66. doi: 10.1016/j.smim.2005.11.002

Facchetti, F., Appiani, C., Salvi, L., Levy, J., & Notarangelo, L. D. (1995). Immunohistologic analysis of ineffective CD40-CD40 ligand interaction in lymphoid tissues from patients with X-linked immunodeficiency with hyper-IgM. Abortive germinal center cell reaction and severe depletion of follicular dendritic cells. J Immunol, 154(12), 6624-6633.

Faller, G., Vollmers, H. P., Weiglein, I., Marx, A., Zink, C., Pfaff, M., & Muller-Hermelink, H. K. (1990). HAB-1, a new heteromyeloma for continuous production of human monoclonal antibodies. Br J Cancer, 62(4), 595-598.

Ferguson, M. A. (1999). The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. J Cell Sci, 112 (Pt 17), 2799-2809.

Ferlay, J., Parkin, D. M., & Steliarova-Foucher, E. (2010). Estimates of cancer incidence and mortality in Europe in 2008. Eur J Cancer, 46(4), 765-781. doi: 10.1016/j.ejca.2009.12.014

Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D., & Anderton, S. M. (2002). B cells regulate autoimmunity by provision of IL-10. Nat Immunol, 3(10), 944-950. doi: 10.1038/ni833

Fishelson, Z., Donin, N., Zell, S., Schultz, S., & Kirschfink, M. (2003). Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Mol Immunol, 40(2-4), 109-123.

Forrester, J. A., Ambrose, E. J., & Macpherson, J. A. (1962). Electrophoretic investigations of a clone of hamster fibroblasts and polyoma-transformed cells from the same population. Nature, 196, 1068-1070.

Forrester, J. A., Ambrose, E. J., & Stoker, M. G. (1964). MICROELECTROPHORESIS OF NORMAL AND TRANSFORMED CLONES OF HAMSTER KIDNEY FIBROBLASTS. Nature, 201, 945-946.

Foy, T. M., Laman, J. D., Ledbetter, J. A., Aruffo, A., Claassen, E., & Noelle, R. J. (1994). gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. [Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. J Exp Med, 180(1), 157-163.

Fredman, P., Hedberg, K., & Brezicka, T. (2003). Gangliosides as therapeutic targets for cancer. BioDrugs, 17(3), 155-167.

French, R. R., Chan, H. T., Tutt, A. L., & Glennie, M. J. (1999). CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. [Research Support, Non-U.S. Gov't]. Nat Med, 5(5), 548-553. doi: 10.1038/8426

Freund, J., Casals, J., & Hosmer, E. P. (1937). Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. Proceedings of the Society for Experimental Biology and Medicine, 37(3), 509-513.

Fujii, S., Shimizu, K., Smith, C., Bonifaz, L., & Steinman, R. M. (2003). Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med, 198(2), 267-279. doi: 10.1084/jem.20030324

Fujimoto, T., Hayashi, M., Iwamoto, M., & Ohno-Iwashita, Y. (1997). Crosslinked plasmalemmal cholesterol is sequestered to caveolae: analysis with a new cytochemical probe. J Histochem Cytochem, 45(9), 1197-1205.

Fujita, A., Cheng, J., Tauchi-Sato, K., Takenawa, T., & Fujimoto, T. (2009). A distinct pool of phosphatidylinositol 4,5-bisphosphate in caveolae revealed by a nanoscale labeling technique. Proc Natl Acad Sci U S A, 106(23), 9256-9261. doi: 10.1073/pnas.0900216106

Fukuda, M. N. (2012). Peptide-displaying phage technology in glycobiology. [Research Support, N.I.H., Extramural

Review]. Glycobiology, 22(3), 318-325. doi: 10.1093/glycob/cwr140

Fukuoka, K., Narita, N., & Saijo, N. (1998). Increased expression of sialyl Lewis(x) antigen is associated with distant metastasis in lung cancer patients: immunohistochemical study on bronchofiberscopic biopsy specimens. [Research Support, Non-U.S. Gov't]. Lung Cancer, 20(2), 109-116.

Fuster, M. M., & Esko, J. D. (2005). The sweet and sour of cancer: glycans as novel therapeutic targets. Nat Rev Cancer, 5(7), 526-542. doi: 10.1038/nrc1649

Futerman, A. H., & Pagano, R. E. (1991). Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver. Biochem J, 280 (Pt 2), 295-302.

Galli, G., Nuti, S., Tavarini, S., Galli-Stampino, L., De Lalla, C., Casorati, G., . . . Abrignani, S. (2003). CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. J Exp Med, 197(8), 1051-1057. doi: 10.1084/jem.20021616

Garrido-Laguna, I., & Hidalgo, M. (2015). Pancreatic cancer: from state-of-the-art treatments to promising novel therapies. Nat Rev Clin Oncol, 12(6), 319-334. doi: 10.1038/nrclinonc.2015.53

Gasic, G., & Gasic, T. (1962). Removal and regeneration of the cell coating in tumour cells. Nature, 196, 170.

Georgopoulos, K. (2002). Haematopoietic cell-fate decisions, chromatin regulation and ikaros. Nat Rev Immunol, 2(3), 162-174. doi: 10.1038/nri747

Gessner, P., Riedl, S., Quentmaier, A., & Kemmner, W. (1993). Enhanced activity of CMP-neuAc:Gal beta 1-4GlcNAc:alpha 2,6-sialyltransferase in metastasizing human colorectal tumor tissue and serum of tumor patients. Cancer Lett, 75(3), 143-149.

Ghosn, E. E., Sadate-Ngatchou, P., Yang, Y., Herzenberg, L. A., & Herzenberg, L. A. (2011). Distinct progenitors for B-1 and B-2 cells are present in adult mouse spleen. Proc Natl Acad Sci U S A, 108(7), 2879-2884. doi: 10.1073/pnas.1019764108

Gillan, V., Lawrence, R. A., & Devaney, E. (2005). B cells play a regulatory role in mice infected with the L3 of Brugia pahangi. Int Immunol, 17(4), 373-382. doi: 10.1093/intimm/dxh217

Gillen, S., Schuster, T., Meyer Zum Buschenfelde, C., Friess, H., & Kleeff, J. (2010). Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. PLoS Med, 7(4), e1000267. doi: 10.1371/journal.pmed.1000267

Godfrey, D. I., MacDonald, H. R., Kronenberg, M., Smyth, M. J., & Van Kaer, L. (2004). NKT cells: what's in a name? Nat Rev Immunol, 4(3), 231-237. doi: 10.1038/nri1309

Godfrey, D. I., Stankovic, S., & Baxter, A. G. (2010). Raising the NKT cell family. Nat Immunol, 11(3), 197-206. doi: 10.1038/ni.1841

Goldberg, M. V., Maris, C. H., Hipkiss, E. L., Flies, A. S., Zhen, L., Tuder, R. M., . . . Drake, C. G. (2007). Role of PD-1 and its ligand, B7-H1, in early fate decisions of CD8 T cells. [Research Support, Non-U.S. Gov't]. Blood, 110(1), 186-192. doi: 10.1182/blood-2006-12-062422

Golfier, S., Kopitz, C., Kahnert, A., Heisler, I., Schatz, C. A., Stelte-Ludwig, B., . . . Ziegelbauer, K. (2014). Anetumab ravtansine: a novel mesothelin-targeting antibody-drug conjugate cures tumors with heterogeneous target expression favored by bystander effect. Mol Cancer Ther, 13(6), 1537-1548. doi: 10.1158/1535-7163.mct-13-0926

Gomez-Vargas, A., Rosenthal, K. L., McDermott, M. R., & Hortelano, G. (2004). Continuous antigenic stimulation system (CASS) as a new immunization strategy. Vaccine, 22(29-30), 3902-3910. doi: 10.1016/j.vaccine.2004.04.010

Gooi, H. C., Feizi, T., Kapadia, A., Knowles, B. B., Solter, D., & Evans, M. J. (1981). Stage-specific embryonic antigen involves alpha 1 goes to 3 fucosylated type 2 blood group chains. Nature, 292(5819), 156-158.

Gorden, P., Carpentier, J. L., Cohen, S., & Orci, L. (1978). Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. Proc Natl Acad Sci U S A, 75(10), 5025-5029.

Gorman, S. D., & Clark, M. R. (1990). Humanisation of monoclonal antibodies for therapy. Semin Immunol, 2(6), 457-466.

Gottlieb, T. M., & Jackson, S. P. (1993). The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell, 72(1), 131-142.

Gottschling, S., Jensen, K., Warth, A., Herth, F. J., Thomas, M., Schnabel, P. A., & Herpel, E. (2013). Stage-specific embryonic antigen-4 is expressed in basaloid lung cancer and associated with poor prognosis. Eur Respir J, 41(3), 656-663. doi: 10.1183/09031936.00225711

Grandal, M. V., Grovdal, L. M., Henriksen, L., Andersen, M. H., Holst, M. R., Madshus, I. H., & van Deurs, B. (2012). Differential roles of Grb2 and AP-2 in p38 MAPK- and EGF-induced EGFR internalization. Traffic, 13(4), 576-585. doi: 10.1111/j.1600-0854.2011.01322.x

Grant, B. D., & Donaldson, J. G. (2009). Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol, 10(9), 597-608. doi: 10.1038/nrm2755

Grant, S. C., Kostakoglu, L., Kris, M. G., Yeh, S. D., Larson, S. M., Finn, R. D., . . . Cheung, N. V. (1996). Targeting of small-cell lung cancer using the anti-GD2 ganglioside monoclonal antibody 3F8: a pilot trial. Eur J Nucl Med, 23(2), 145-149.

Greenspan, N. S., & Cooper, L. J. (1993). Cooperative binding by mouse IgG3 antibodies: implications for functional affinity, effector function, and isotype restriction. Springer Semin Immunopathol, 15(2-3), 275-291.

Greenspan, N. S., Monafo, W. J., & Davie, J. M. (1987). Interaction of IgG3 anti-streptococcal group A carbohydrate (GAC) antibody with streptococcal group A vaccine: enhancing and inhibiting effects of anti-GAC, anti-isotypic, and anti-idiotypic antibodies. J Immunol, 138(1), 285-292.

Grey, H. M., Hirst, J. W., & Cohn, M. (1971). A new mouse immunoglobulin: IgG3. J Exp Med, 133(2), 289-304.

Griffiths, G. M., Berek, C., Kaartinen, M., & Milstein, C. (1984). Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. Nature, 312(5991), 271-275.

Grimaldi, C. M., & French, D. L. (1995). Monoclonal Antibodies by Somatic Cell Fusion. ILAR J, 37(3), 125-132.

Grimbacher, B., Hutloff, A., Schlesier, M., Glocker, E., Warnatz, K., Drager, R., . . . Peter, H. H. (2003). Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol, 4(3), 261-268. doi: 10.1038/ni902

Guan, Z. Z., Xu, J. M., Luo, R. C., Feng, F. Y., Wang, L. W., Shen, L., . . . Xu, R. H. (2011). Efficacy and safety of bevacizumab plus chemotherapy in Chinese patients with metastatic colorectal cancer: a randomized phase III ARTIST trial. Chin J Cancer, 30(10), 682-689. doi: 10.5732/cjc.011.10188

Guo, Y., Ma, J., Wang, J., Che, X., Narula, J., Bigby, M., . . . Sy, M. S. (1994). Inhibition of human melanoma growth and metastasis in vivo by anti-CD44 monoclonal antibody. Cancer Res, 54(6), 1561-1565.

Gupta, R. K. (1998). Aluminum compounds as vaccine adjuvants. Adv Drug Deliv Rev, 32(3), 155-172.

Guy, B. (2007). The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol, 5(7), 505-517. doi: 10.1038/nrmicro1681

Gyotoku, Y., Abdelmoula, M., Spertini, F., Izui, S., & Lambert, P. H. (1987). Cryoglobulinemia induced by monoclonal immunoglobulin G rheumatoid factors derived from autoimmune MRL/MpJ-lpr/lpr mice. J Immunol, 138(11), 3785-3792.

Hafner, C., Wagner, S., Jasinska, J., Allwardt, D., Scheiner, O., Wolff, K., . . . Breiteneder, H. (2005). Epitope-specific antibody response to Mel-CAM induced by mimotope immunization. [Research Support, Non-U.S. Gov't]. J Invest Dermatol, 124(1), 125-131. doi: 10.1111/j.0022-202X.2004.23515.x

Hagan, S., Orr, M. C., & Doyle, B. (2013). Targeted therapies in colorectal cancer-an integrative view by PPPM. EPMA J, 4(1), 3. doi: 10.1186/1878-5085-4-3

Hagemann, T., Balkwill, F., & Lawrence, T. (2007). Inflammation and cancer: a double-edged sword. Cancer Cell, 12(4), 300-301. doi: 10.1016/j.ccr.2007.10.005

Hainsworth, J. D., Vazquez, E. R., Spigel, D. R., Raefsky, E., Bearden, J. D., Saez, R. A., & Greco, F. A. (2008). Combination therapy with fludarabine and rituximab followed by alemtuzumab in the first-line treatment of patients with chronic lymphocytic leukemia or small lymphocytic lymphoma: a phase 2 trial of the Minnie Pearl Cancer Research Network. [Clinical Trial, Phase II

Multicenter Study

Research Support, Non-U.S. Gov't]. Cancer, 112(6), 1288-1295. doi: 10.1002/cncr.23271

Hakomori, S. (2002). Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci U S A, 99(16), 10231-10233. doi: 10.1073/pnas.172380699

Hakomori, S. (2004). Glycosynapses: microdomains controlling carbohydrate-dependent cell adhesion and signaling. An Acad Bras Cienc, 76(3), 553-572. doi: /S0001-37652004000300010

Hakomori, S., & Andrews, H. D. (1970). Sphingoglycolipids with Leb activity, and the co-presence of Lea-, Leb-glycolipids in human tumor tissue. Biochim Biophys Acta, 202(1), 225-228.

Hakomori, S., & Kannagi, R. (1983). Glycosphingolipids as tumor-associated and differentiation markers. J Natl Cancer Inst, 71(2), 231-251.

Hakomori, S. I. (2008). Structure and function of glycosphingolipids and sphingolipids: recollections and future trends. Biochim Biophys Acta, 1780(3), 325-346. doi: 10.1016/j.bbagen.2007.08.015

Hamid, O., Robert, C., Daud, A., Hodi, F. S., Hwu, W. J., Kefford, R., . . . Ribas, A. (2013). Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. [Clinical Trial

Research Support, Non-U.S. Gov't]. N Engl J Med, 369(2), 134-144. doi: 10.1056/NEJMoa1305133

Hammarlund, E., Lewis, M. W., Hansen, S. G., Strelow, L. I., Nelson, J. A., Sexton, G. J., . . . Slifka, M. K. (2003). Duration of antiviral immunity after smallpox vaccination. Nat Med, 9(9), 1131-1137. doi: 10.1038/nm917

Han, X., Papadopoulos, A. J., Ruparelia, V., Devaja, O., & Raju, K. S. (1997). Tumor lymphocytes in patients with advanced ovarian cancer: changes during in vitro culture and implications for immunotherapy. Gynecol Oncol, 65(3), 391-398. doi: 10.1006/gyno.1997.4668

Handa, K., & Hakomori, S. I. (2012). Carbohydrate to carbohydrate interaction in development process and cancer progression. Glycoconj J, 29(8-9), 627-637. doi: 10.1007/s10719-012-9380-7

Harder, T., Scheiffele, P., Verkade, P., & Simons, K. (1998). Lipid domain structure of the plasma membrane revealed by patching of membrane components. J Cell Biol, 141(4), 929-942.

Harduin-Lepers, A., Vallejo-Ruiz, V., Krzewinski-Recchi, M. A., Samyn-Petit, B., Julien, S., & Delannoy, P. (2001). The human sialyltransferase family. [Research Support, Non-U.S. Gov't

Review]. Biochimie, 83(8), 727-737.

Hardy, R. R. (2006). B-1 B cell development. J Immunol, 177(5), 2749-2754.

Harris, R. J., Shire, S. J., & Winter, C. (2004). Commerical manufacturing scale formulation and analytical characterisation of therapeutic recombinant antibodies. Drug Development Research, 61(3), 137-154.

Hassan, S. B., Sorensen, J. F., Olsen, B. N., & Pedersen, A. E. (2014). Anti-CD40-mediated cancer immunotherapy: an update of recent and ongoing clinical trials. [Review]. Immunopharmacol Immunotoxicol, 36(2), 96-104. doi: 10.3109/08923973.2014.890626

Hayes, J. M., Cosgrave, E. F., Struwe, W. B., Wormald, M., Davey, G. P., Jefferis, R., & Rudd, P. M. (2014). Glycosylation and Fc receptors. Curr Top Microbiol Immunol, 382, 165-199. doi: 10.1007/978-3-319-07911-0_8

Haynes, P. A. (1998). Phosphoglycosylation: a new structural class of glycosylation? Glycobiology, 8(1), 1-5.

He, J., & Efron, J. E. (2011). Screening for colorectal cancer. Adv Surg, 45, 31-44.

Hedlund, M., Ng, E., Varki, A., & Varki, N. M. (2008). alpha 2-6-Linked sialic acids on N-glycans modulate carcinoma differentiation in vivo. Cancer Res, 68(2), 388-394. doi: 10.1158/0008-5472.can-07-1340

Heffer-Lauc, M., Lauc, G., Nimrichter, L., Fromholt, S. E., & Schnaar, R. L. (2005). Membrane redistribution of gangliosides and glycosylphosphatidylinositol-anchored proteins in brain tissue sections under conditions of lipid raft isolation. Biochim Biophys Acta, 1686(3), 200-208. doi: 10.1016/j.bbalip.2004.10.002

Heimburg-Molinaro, J., Lum, M., Vijay, G., Jain, M., Almogren, A., & Rittenhouse-Olson, K. (2011). Cancer vaccines and carbohydrate epitopes. Vaccine, 29(48), 8802-8826. doi: 10.1016/j.vaccine.2011.09.009

Helft, P. R., Schilsky, R. L., Hoke, F. J., Williams, D., Kindler, H. L., Sprague, E., . . . Ratain, M. J. (2004). A phase I study of cantuzumab mertansine administered as a single intravenous infusion once weekly in patients with advanced solid tumors. [Clinical Trial

Clinical Trial, Phase I

Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. Clin Cancer Res, 10(13), 4363-4368. doi: 10.1158/1078-0432.CCR-04-0088

Hellstrom, I., Garrigues, H. J., Garrigues, U., & Hellstrom, K. E. (1990). Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le(y)-related cell surface antigens. Cancer Res, 50(7), 2183-2190.

Henderson, J. K., Draper, J. S., Baillie, H. S., Fishel, S., Thomson, J. A., Moore, H., & Andrews, P. W. (2002). Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. [Comparative Study

Research Support, Non-U.S. Gov't]. Stem Cells, 20(4), 329-337. doi: 10.1634/stemcells.20-4-329

Henriksen-Lacey, M., Bramwell, V. W., Christensen, D., Agger, E. M., Andersen, P., & Perrie, Y. (2010). Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. J Control Release, 142(2), 180-186. doi: 10.1016/j.jconrel.2009.10.022

Henriksen, L., Grandal, M. V., Knudsen, S. L., van Deurs, B., & Grovdal, L. M. (2013). Internalization mechanisms of the epidermal growth factor receptor after activation with different ligands. PLoS One, 8(3), e58148. doi: 10.1371/journal.pone.0058148

Hernandez, A. M., Rodriguez, N., Gonzalez, J. E., Reyes, E., Rondon, T., Grinan, T., . . . Perez, R. (2011). Anti-NeuGcGM3 antibodies, actively elicited by idiotypic vaccination in nonsmall cell lung cancer patients, induce tumor cell death by an oncosis-like mechanism. J Immunol, 186(6), 3735-3744. doi: 10.4049/jimmunol.1000609

Herrmann, R., Bodoky, G., Ruhstaller, T., Glimelius, B., Saletti, P., Bajetta, E., . . . Scheithauer, W. (2005). Gemcitabine (G) plus capecitabine (C) versus G alone in locally advanced or metastatic pancreatic cancer. A randomized phase III study of the Swiss Group for Clinical Cancer Research (SAKK) and the Central European Cooperative Oncology Group (CECOG). Journal of Clinical Oncology, 23(16), 310s-310s.

Herter, S., Birk, M. C., Klein, C., Gerdes, C., Umana, P., & Bacac, M. (2014). Glycoengineering of therapeutic antibodies enhances monocyte/macrophage-mediated phagocytosis and cytotoxicity. J Immunol, 192(5), 2252-2260. doi: 10.4049/jimmunol.1301249

Herzenberg, L. A. (2000). B-1 cells: the lineage question revisited. Immunol Rev, 175, 9-22.

Herzenberg, L. A., Tokuhisa, T., & Herzenberg, L. A. (1980). Carrier-priming leads to hapten-specific suppression. Nature, 285(5767), 664-667.

Herzenberg, L. A., & Tung, J. W. (2006). B cell lineages: documented at last! Nat Immunol, 7(3), 225-226. doi: 10.1038/ni0306-225

Hess, J., Werner, A., Wirth, T., Melchers, F., Jack, H. M., & Winkler, T. H. (2001). Induction of pre-B cell proliferation after de novo synthesis of the pre-B cell receptor. Proc Natl Acad Sci U S A, 98(4), 1745-1750. doi: 10.1073/pnas.041492098

Hidalgo, M., Cascinu, S., Kleeff, J., Labianca, R., Lohr, J. M., Neoptolemos, J., . . . Heinemann, V. (2015). Addressing the challenges of pancreatic cancer: future directions for improving outcomes. Pancreatology, 15(1), 8-18. doi: 10.1016/j.pan.2014.10.001

Hillion, S., Rochas, C., Youinou, P., & Jamin, C. (2009). Signaling pathways regulating RAG expression in B lymphocytes. Autoimmun Rev, 8(7), 599-604. doi: 10.1016/j.autrev.2009.02.004

Hirst, T. R., Fraser, S., Soriani, M., Aman, A. T., de, H. L., Hearn, A., & Merritt, E. (2002). New insights into the structure-function relationships and therapeutic applications of cholera-like enterotoxins. Int J Med Microbiol, 291(6-7), 531-535.

Ho-Yen, C. M., Green, A. R., Rakha, E. A., Brentnall, A. R., Ellis, I. O., Kermorgant, S., & Jones, J. L. (2014). C-Met in invasive breast cancer: is there a relationship with the basal-like subtype? Cancer, 120(2), 163-171. doi: 10.1002/cncr.28386

Hodi, F. S., O'Day, S. J., McDermott, D. F., Weber, R. W., Sosman, J. A., Haanen, J. B., . . . Urba, W. J. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. [Clinical Trial, Phase III

Comparative Study

Multicenter Study

Randomized Controlled Trial]. N Engl J Med, 363(8), 711-723. doi: 10.1056/NEJMoa1003466

Hodoniczky, J., Zheng, Y. Z., & James, D. C. (2005). Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro. [Research Support, Non-U.S. Gov't]. Biotechnol Prog, 21(6), 1644-1652. doi: 10.1021/bp050228w

Hoess, R., Brinkmann, U., Handel, T., & Pastan, I. (1993). Identification of a peptide which binds to the carbohydrate-specific monoclonal antibody B3. Gene, 128(1), 43-49.

Hoet, R. M., Cohen, E. H., Kent, R. B., Rookey, K., Schoonbroodt, S., Hogan, S., . . . Ladner, R. C. (2005). Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. Nat Biotechnol, 23(3), 344-348. doi: 10.1038/nbt1067

Hofsteenge, J., Muller, D. R., de Beer, T., Loffler, A., Richter, W. J., & Vliegenthart, J. F. (1994). New type of linkage between a carbohydrate and a protein: C-glycosylation of a specific tryptophan residue in human RNase Us. Biochemistry, 33(46), 13524-13530.

Hollingsworth, M. A., & Swanson, B. J. (2004). Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer, 4(1), 45-60. doi: 10.1038/nrc1251

Holt, L. J., Herring, C., Jespers, L. S., Woolven, B. P., & Tomlinson, I. M. (2003). Domain antibodies: proteins for therapy. Trends Biotechnol, 21(11), 484-490. doi: 10.1016/j.tibtech.2003.08.007

Huang, Y. L., Hung, J. T., Cheung, S. K., Lee, H. Y., Chu, K. C., Li, S. T., . . . Wong, C. H. (2013). Carbohydrate-based vaccines with a glycolipid adjuvant for breast cancer. Proc Natl Acad Sci U S A, 110(7), 2517-2522. doi: 10.1073/pnas.1222649110

Hughes, E. A., & Cresswell, P. (1998). The thiol oxidoreductase ERp57 is a component of the MHC class I peptide-loading complex. Curr Biol, 8(12), 709-712.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., . . . Kabbinavar, F. (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med, 350(23), 2335-2342. doi: 10.1056/NEJMoa032691

Hust, M., & Dubel, S. (2005). Phage display vectors for the in vitro generation of human antibody fragments. Methods Mol Biol, 295, 71-96.

Hutloff, A., Dittrich, A. M., Beier, K. C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., & Kroczek, R. A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature, 397(6716), 263-266. doi: 10.1038/16717

Huttinger, R., Staffler, G., Majdic, O., & Stockinger, H. (1999). Analysis of the early biogenesis of CD1b: involvement of the chaperones calnexin and calreticulin, the proteasome and beta(2)-microglobulin. Int Immunol, 11(10), 1615-1623.

Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K. I., & Hirabayashi, Y. (1996). Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis. Proc Natl Acad Sci U S A, 93(10), 4638-4643.

Idusogie, E. E., Wong, P. Y., Presta, L. G., Gazzano-Santoro, H., Totpal, K., Ultsch, M., & Mulkerrin, M. G. (2001). Engineered antibodies with increased activity to recruit complement. J Immunol, 166(4), 2571-2575.

Imberty, A., Mikros, E., Koca, J., Mollicone, R., Oriol, R., & Perez, S. (1995). Computer simulation of histo-blood group oligosaccharides: energy maps of all constituting disaccharides and potential energy surfaces of 14 ABH and Lewis carbohydrate antigens. [Research Support, Non-U.S. Gov't]. Glycoconj J, 12(3), 331-349.

Inagaki, H., Sakamoto, J., Nakazato, H., Bishop, A. E., & Yura, J. (1990). Expression of Lewis(a), Lewis(b), and sialated Lewis(a) antigens in early and advanced human gastric cancers. J Surg Oncol, 44(4), 208-213.

Inagaki, Y., Gao, J., Song, P., Kokudo, N., Nakata, M., & Tang, W. (2014). Clinicopathological utility of sialoglycoconjugates in diagnosing and treating colorectal cancer. World J Gastroenterol, 20(20), 6123-6132. doi: 10.3748/wjg.v20.i20.6123

Inal, A., Kos, F. T., Algin, E., Yildiz, R., Dikiltas, M., Unek, I. T., . . . Isikdogan, A. (2012). Gemcitabine alone versus combination of gemcitabine and cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic carcinoma: a retrospective analysis of multicenter study. Neoplasma, 59(3), 297-301. doi: 10.4149/neo_2012_038

Intlekofer, A. M., & Thompson, C. B. (2013). At the bench: preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy. [Research Support, N.I.H., Extramural

Review]. J Leukoc Biol, 94(1), 25-39. doi: 10.1189/jlb.1212621

Isozaki, H., Ohyama, T., & Mabuchi, H. (1998). Expression of cell adhesion molecule CD44 and sialyl Lewis A in gastric carcinoma and colorectal carcinoma in association with hepatic metastasis. Int J Oncol, 13(5), 935-942.

Jacob, J., Kelsoe, G., Rajewsky, K., & Weiss, U. (1991). Intraclonal generation of antibody mutants in germinal centres. Nature, 354(6352), 389-392. doi: 10.1038/354389a0

Jeckel, D., Karrenbauer, A., Burger, K. N., van Meer, G., & Wieland, F. (1992). Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. J Cell Biol, 117(2), 259-267.

Jefferis, R. (2009a). Aglycosylated antibodies and the methods of making and using them: WO2008030564. Expert Opin Ther Pat, 19(1), 101-105. doi: 10.1517/13543770802592408

Jefferis, R. (2009b). Glycosylation as a strategy to improve antibody-based therapeutics. [Review]. Nat Rev Drug Discov, 8(3), 226-234. doi: 10.1038/nrd2804

Jefferis, R. (2009c). Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. [Review]. Trends Pharmacol Sci, 30(7), 356-362. doi: 10.1016/j.tips.2009.04.007

Jefferis, R., & Lund, J. (2002). Interaction sites on human IgG-Fc for FcgammaR: current models. [Review]. Immunol Lett, 82(1-2), 57-65.

Jennings, V. M. (1995). Review of Selected Adjuvants Used in Antibody Production. ILAR J, 37(3), 119-125.

Jewett, A., & Tseng, H. C. (2011). Tumor induced inactivation of natural killer cell cytotoxic function; implication in growth, expansion and differentiation of cancer stem cells. J Cancer, 2, 443-457.

Jiang, X. R., Song, A., Bergelson, S., Arroll, T., Parekh, B., May, K., . . . Schenerman, M. (2011). Advances in the assessment and control of the effector functions of therapeutic antibodies. Nat Rev Drug Discov, 10(2), 101-111. doi: 10.1038/nrd3365

Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S., & Winter, G. (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature, 321(6069), 522-525. doi: 10.1038/321522a0

Josephs, D. H., Bax, H. J., & Karagiannis, S. N. (2015). Tumour-associated macrophage polarisation and re-education with immunotherapy. [Research Support, Non-U.S. Gov't

Review]. Front Biosci (Elite Ed), 7, 293-308.

Julien, S., Adriaenssens, E., Ottenberg, K., Furlan, A., Courtand, G., Vercoutter-Edouart, A. S., . . . Le Bourhis, X. (2006). ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology, 16(1), 54-64. doi: 10.1093/glycob/cwj033

Julien, S., Ivetic, A., Grigoriadis, A., QiZe, D., Burford, B., Sproviero, D., . . . Burchell, J. M. (2011). Selectin ligand sialyl-Lewis x antigen drives metastasis of hormone-dependent breast cancers. Cancer Res, 71(24), 7683-7693. doi: 10.1158/0008-5472.can-11-1139

Kaisho, T., & Akira, S. (2002). Toll-like receptors as adjuvant receptors. Biochim Biophys Acta, 1589(1), 1-13.

Kanda, Y., Yamada, T., Mori, K., Okazaki, A., Inoue, M., Kitajima-Miyama, K., . . . Satoh, M. (2007). Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types. Glycobiology, 17(1), 104-118. doi: 10.1093/glycob/cwl057

Kang, S. J., & Cresswell, P. (2002). Calnexin, calreticulin, and ERp57 cooperate in disulfide bond formation in human CD1d heavy chain. J Biol Chem, 277(47), 44838-44844. doi: 10.1074/jbc.M207831200

Kannagi, R. (2007). Carbohydrate antigen sialyl Lewis a--its pathophysiological significance and induction mechanism in cancer progression. Chang Gung Med J, 30(3), 189-209.

Kannagi, R., Cochran, N. A., Ishigami, F., Hakomori, S., Andrews, P. W., Knowles, B. B., & Solter, D. (1983). Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. EMBO J, 2(12), 2355-2361.

Kannagi, R., Izawa, M., Koike, T., Miyazaki, K., & Kimura, N. (2004). Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. Cancer Sci, 95(5), 377-384.

Kantele, A., Hakkinen, M. P., Zivny, J., Elson, C. O., Mestecky, J., & Kantele, J. M. (2011). Humoral immune response to keyhole limpet haemocyanin, the protein carrier in cancer vaccines. Clin Dev Immunol, 2011, 614383. doi: 10.1155/2011/614383

Karasuyama, H., Rolink, A., Shinkai, Y., Young, F., Alt, F. W., & Melchers, F. (1994). The expression of Vpre-B/lambda 5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. Cell, 77(1), 133-143.

Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., . . . Taniguchi, M. (1997). CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science, 278(5343), 1626-1629.

Kawano, T., Yanoma, S., Nakamura, Y., Shiono, O., Kokatu, T., Kubota, A., . . . Tsukuda, M. (2005). Evaluation of soluble adhesion molecules CD44 (CD44st, CD44v5, CD44v6), ICAM-1, and VCAM-1 as tumor markers in head and neck cancer. Am J Otolaryngol, 26(5), 308-313. doi: 10.1016/j.amjoto.2005.02.005

Kazazic, M., Roepstorff, K., Johannessen, L. E., Pedersen, N. M., van Deurs, B., Stang, E., & Madshus, I. H. (2006). EGF-induced activation of the EGF receptor does not trigger mobilization of caveolae. Traffic, 7(11), 1518-1527. doi: 10.1111/j.1600-0854.2006.00487.x

Kellokumpu, S., Sormunen, R., & Kellokumpu, I. (2002). Abnormal glycosylation and altered Golgi structure in colorectal cancer: dependence on intra-Golgi pH. FEBS Lett, 516(1-3), 217-224.

Kerzerho, J., Yu, E. D., Barra, C. M., Alari-Pahissa, E., Girardi, E., Harrak, Y., . . . Castano, A. R. (2012). Structural and functional characterization of a novel nonglycosidic type I NKT agonist with immunomodulatory properties. J Immunol, 188(5), 2254-2265. doi: 10.4049/jimmunol.1103049

Keusch, G. T., Jacewicz, M., Mobassaleh, M., & Donohue-Rolfe, A. (1991). Shiga toxin: intestinal cell receptors and pathophysiology of enterotoxic effects. Rev Infect Dis, 13 Suppl 4, S304-310.

Khalil, M., & Vonderheide, R. H. (2007). Anti-CD40 agonist antibodies: preclinical and clinical experience. Update Cancer Ther, 2(2), 61-65. doi: 10.1016/j.uct.2007.06.001

Kieber-Emmons, T., Luo, P., Qiu, J., Chang, T. Y., O, I., Blaszczyk-Thurin, M., & Steplewski, Z. (1999). Vaccination with carbohydrate peptide mimotopes promotes anti-tumor responses. [Research Support, U.S. Gov't, Non-P.H.S.

Research Support, U.S. Gov't, P.H.S.]. Nat Biotechnol, 17(7), 660-665. doi: 10.1038/10870

Kim, M. J., Kim, H. S., Song, K. S., Noh, S. H., Kim, H. G., Paik, Y. K., & Kim, H. O. (2002). Altered expression of Lewis antigen on tissue and erythrocytes in gastric cancer patients. Yonsei Med J, 43(4), 427-434.

Kim, S., Lalani, S., Parekh, V. V., Wu, L., & Van Kaer, L. (2008). Glycolipid ligands of invariant natural killer T cells as vaccine adjuvants. Expert Rev Vaccines, 7(10), 1519-1532. doi: 10.1586/14760584.7.10.1519

Kim, Y. J., & Varki, A. (1997). Perspectives on the significance of altered glycosylation of glycoproteins in cancer. Glycoconj J, 14(5), 569-576.

Kobayashi, H., Mochizuki, H., Morita, T., Kotake, K., Teramoto, T., Kameoka, S., . . . Sugihara, K. (2011). Characteristics of recurrence after curative resection for T1 colorectal cancer: Japanese multicenter study. J Gastroenterol, 46(2), 203-211. doi: 10.1007/s00535-010-0341-2

Kobayashi, M., Mizuno, S., Murata, Y., Kishiwada, M., Usui, M., Sakurai, H., . . . Isaji, S. (2014). Gemcitabine-based chemoradiotherapy followed by surgery for borderline resectable and locally unresectable pancreatic ductal adenocarcinoma: significance of the CA19-9 reduction rate and intratumoral human equilibrative nucleoside transporter 1 expression. Pancreas, 43(3), 350-360. doi: 10.1097/MPA.0000000000000059

Koch, M., Stronge, V. S., Shepherd, D., Gadola, S. D., Mathew, B., Ritter, G., . . . Cerundolo, V. (2005). The crystal structure of human CD1d with and without alpha-galactosylceramide. [Research Support, Non-U.S. Gov't]. Nat Immunol, 6(8), 819-826. doi: 10.1038/ni1225

Koh, Y. W., Lee, H. J., Ahn, J. H., Lee, J. W., & Gong, G. (2013). Expression of Lewis X is associated with poor prognosis in triple-negative breast cancer. Am J Clin Pathol, 139(6), 746-753. doi: 10.1309/ajcp2e6qndidpttc

Kohler, G., & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256(5517), 495-497.

Kohls, M. D., & Lappi, D. A. (2000). Mab-ZAP: a tool for evaluating antibody efficacy for use in an immunotoxin. Biotechniques, 28(1), 162-165.

Kojima, A., Iwata, K., Seya, T., Matsumoto, M., Ariga, H., Atkinson, J. P., & Nagasawa, S. (1993). Membrane cofactor protein (CD46) protects cells predominantly from alternative complement pathway-mediated C3-fragment deposition and cytolysis. J Immunol, 151(3), 1519-1527.

Koprowski, H., Steplewski, Z., Mitchell, K., Herlyn, M., Herlyn, D., & Fuhrer, P. (1979). Colorectal carcinoma antigens detected by hybridoma antibodies. [Research Support, U.S. Gov't, P.H.S.]. Somatic Cell Genet, 5(6), 957-971.

Koretz, K., Bruderlein, S., Henne, C., & Moller, P. (1993). Expression of CD59, a complement regulator protein and a second ligand of the CD2 molecule, and CD46 in normal and neoplastic colorectal epithelium. Br J Cancer, 68(5), 926-931.

Kovtun, Y. V., Audette, C. A., Ye, Y., Xie, H., Ruberti, M. F., Phinney, S. J., . . . Goldmacher, V. S. (2006). Antibody-drug conjugates designed to eradicate tumors with homogeneous and heterogeneous expression of the target antigen. Cancer Res, 66(6), 3214-3221. doi: 10.1158/0008-5472.can-05-3973

Kris, M. G., Natale, R. B., Herbst, R. S., Lynch, T. J., Jr., Prager, D., Belani, C. P., . . . Kay, A. C. (2003). Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA, 290(16), 2149-2158. doi: 10.1001/jama.290.16.2149

Kristiansen, G., Sammar, M., & Altevogt, P. (2004). Tumour biological aspects of CD24, a mucin-like adhesion molecule. J Mol Histol, 35(3), 255-262.

Kronenberg, M. (2005). Toward an understanding of NKT cell biology: progress and paradoxes. [Research Support, U.S. Gov't, P.H.S.

Review]. Annu Rev Immunol, 23, 877-900. doi: 10.1146/annurev.immunol.23.021704.115742

Kroon, D. J., Baldwin-Ferro, A., & Lalan, P. (1992). Identification of sites of degradation in a therapeutic monoclonal antibody by peptide mapping. Pharm Res, 9(11), 1386-1393.

Krop, I. E., Lin, N. U., Blackwell, K., Guardino, E., Huober, J., Lu, M., . . . Dieras, V. (2015). Trastuzumab emtansine (T-DM1) versus lapatinib plus capecitabine in patients with HER2-positive metastatic breast cancer and central nervous system metastases: a retrospective, exploratory analysis in EMILIA. Ann Oncol, 26(1), 113-119. doi: 10.1093/annonc/mdu486

Krug, L. M., Milton, D. T., Jungbluth, A. A., Chen, L. C., Quaia, E., Pandit-Taskar, N., . . . Divgi, C. (2007). Targeting Lewis Y (Le(y)) in small cell lung cancer with a humanized monoclonal antibody, hu3S193: a pilot trial testing two dose levels. J Thorac Oncol, 2(10), 947-952. doi: 10.1097/JTO.0b013e3181560dcc

Krysko, O., Love Aaes, T., Bachert, C., Vandenabeele, P., & Krysko, D. V. (2013). Many faces of DAMPs in cancer therapy. Cell Death Dis, 4, e631. doi: 10.1038/cddis.2013.156

Kubota, T., Niwa, R., Satoh, M., Akinaga, S., Shitara, K., & Hanai, N. (2009). Engineered therapeutic antibodies with improved effector functions. [Review]. Cancer Sci, 100(9), 1566-1572. doi: 10.1111/j.1349-7006.2009.01222.x

Kumamoto, K., Goto, Y., Sekikawa, K., Takenoshita, S., Ishida, N., Kawakita, M., & Kannagi, R. (2001). Increased expression of UDP-galactose transporter messenger RNA in human colon cancer tissues and its implication in synthesis of Thomsen-Friedenreich antigen and sialyl Lewis A/X determinants. Cancer Res, 61(11), 4620-4627.

Kumar, S., Mahdi, H., Bryant, C., Shah, J. P., Garg, G., & Munkarah, A. (2010). Clinical trials and progress with paclitaxel in ovarian cancer. Int J Womens Health, 2, 411-427. doi: 10.2147/ijwh.s7012

Kurman, R. J., & Shih Ie, M. (2010). The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. Am J Surg Pathol, 34(3), 433-443. doi: 10.1097/PAS.0b013e3181cf3d79

Kurosaki, T. (1999). Genetic analysis of B cell antigen receptor signaling. Annu Rev Immunol, 17, 555-592. doi: 10.1146/annurev.immunol.17.1.555

Lambert, J. M., & Chari, R. V. (2014). Ado-trastuzumab Emtansine (T-DM1): an antibody-drug conjugate (ADC) for HER2-positive breast cancer. J Med Chem, 57(16), 6949-6964. doi: 10.1021/jm500766w

Lanzavecchia, A. (1985). Antigen-specific interaction between T and B cells. [Research Support, Non-U.S. Gov't]. Nature, 314(6011), 537-539.

Leadbetter, E. A., Brigl, M., Illarionov, P., Cohen, N., Luteran, M. C., Pillai, S., . . . Brenner, M. B. (2008). NK T cells provide lipid antigen-specific cognate help for B cells. [Research Support, N.I.H., Extramural]. Proc Natl Acad Sci U S A, 105(24), 8339-8344. doi: 10.1073/pnas.0801375105

Lee, J. K., Seki, N., Sayers, T. J., Subleski, J., Gruys, E. M., Murphy, W. J., & Wiltrout, R. H. (2005). Constitutive expression of functional CD40 on mouse renal cancer cells: induction of Fas and Fas-mediated killing by CD40L. Cell Immunol, 235(2), 145-152. doi: 10.1016/j.cellimm.2005.08.029

Lewis Phillips, G. D., Li, G., Dugger, D. L., Crocker, L. M., Parsons, K. L., Mai, E., . . . Sliwkowski, M. X. (2008). Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-

cytotoxic drug conjugate. Cancer Res, 68(22), 9280-9290. doi: 10.1158/0008-5472.can-08-1776

Li, F., Vijayasankaran, N., Shen, A. Y., Kiss, R., & Amanullah, A. (2010). Cell culture processes for monoclonal antibody production. MAbs, 2(5), 466-479.

Li, W., Ran, Y., Li, M., Zhang, K., Qin, X., Xue, X., . . . Zhang, Y. (2013). Mimotope vaccination for epitope-specific induction of anti-VEGF antibodies. [Research Support, Non-U.S. Gov't]. BMC Biotechnol, 13, 77. doi: 10.1186/1472-6750-13-77

Li, Z., Woo, C. J., Iglesias-Ussel, M. D., Ronai, D., & Scharff, M. D. (2004). The generation of antibody diversity through somatic hypermutation and class switch recombination. Genes Dev, 18(1), 1-11. doi: 10.1101/gad.1161904

Liang, Y. J., Kuo, H. H., Lin, C. H., Chen, Y. Y., Yang, B. C., Cheng, Y. Y., . . . Yu, J. (2010). Switching of the core structures of glycosphingolipids from globo- and lacto- to ganglio-series upon human embryonic stem cell differentiation. Proc Natl Acad Sci U S A, 107(52), 22564-22569. doi: 10.1073/pnas.1007290108

Liao, Q., Zhao, Y. P., Yang, Y. C., Li, L. J., Long, X., & Han, S. M. (2007). Combined detection of serum tumor markers for differential diagnosis of solid lesions located at the pancreatic head. [Research Support, Non-U.S. Gov't]. Hepatobiliary Pancreat Dis Int, 6(6), 641-645.

Lieber, M. R., Grawunder, U., Wu, X., & Yaneva, M. (1997). Tying loose ends: roles of Ku and DNA-dependent protein kinase in the repair of double-strand breaks. Curr Opin Genet Dev, 7(1), 99-104.

Lin, C. C., Calvo, E., Papadopoulos, K. P., Patnaik, A., Sarantopoulos, J., Mita, A. C., . . . Tolcher, A. W. (2009). Phase I study of cetuximab, erlotinib, and bevacizumab in patients with advanced solid tumors. Cancer Chemother Pharmacol, 63(6), 1065-1071. doi: 10.1007/s00280-008-0811-x

Lingwood, C. A. (2011). Glycosphingolipid functions. Cold Spring Harb Perspect Biol, 3(7). doi: 10.1101/cshperspect.a004788

Linsley, P. S., Bradshaw, J., Greene, J., Peach, R., Bennett, K. L., & Mittler, R. S. (1996). Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. Immunity, 4(6), 535-543.

Lipman, N. S., Jackson, L. R., Trudel, L. J., & Weis-Garcia, F. (2005). Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. ILAR J, 46(3), 258-268.

Little, M., Kipriyanov, S. M., Le Gall, F., & Moldenhauer, G. (2000). Of mice and men: hybridoma and recombinant antibodies. Immunol Today, 21(8), 364-370.

Liu, J. K. (2014). The history of monoclonal antibody development - Progress, remaining challenges and future innovations. Ann Med Surg (Lond), 3(4), 113-116. doi: 10.1016/j.amsu.2014.09.001

Livingston, P. O., Hood, C., Krug, L. M., Warren, N., Kris, M. G., Brezicka, T., & Ragupathi, G. (2005). Selection of GM2, fucosyl GM1, globo H and polysialic acid as targets on small cell lung cancers for antibody mediated immunotherapy. Cancer Immunol Immunother, 54(10), 1018-1025. doi: 10.1007/s00262-005-0663-8

Loffert, D., Ehlich, A., Muller, W., & Rajewsky, K. (1996). Surrogate light chain expression is required to establish immunoglobulin heavy chain allelic exclusion during early B cell development. Immunity, 4(2), 133-144.

Lonberg, N. (2008). Human monoclonal antibodies from transgenic mice. Handb Exp Pharmacol(181), 69-97. doi: 10.1007/978-3-540-73259-4_4

Loo, D., Pryer, N., Young, P., Liang, T., Coberly, S., King, K. L., . . . Mather, J. P. (2007). The glycotope-specific RAV12 monoclonal antibody induces oncosis in vitro and has antitumor activity against gastrointestinal adenocarcinoma tumor xenografts in vivo. Mol Cancer Ther, 6(3), 856-865. doi: 10.1158/1535-7163.mct-06-0581

Lopez, P. H., & Schnaar, R. L. (2009). Gangliosides in cell recognition and membrane protein regulation. Curr Opin Struct Biol, 19(5), 549-557. doi: 10.1016/j.sbi.2009.06.001

Lopus, M., Oroudjev, E., Wilson, L., Wilhelm, S., Widdison, W., Chari, R., & Jordan, M. A. (2010). Maytansine and cellular metabolites of antibody-maytansinoid conjugates strongly suppress microtubule dynamics by binding to microtubules. Mol Cancer Ther, 9(10), 2689-2699. doi: 10.1158/1535-7163.MCT-10-0644

Lou, Y. W., Wang, P. Y., Yeh, S. C., Chuang, P. K., Li, S. T., Wu, C. Y., . . . Wong, C. H. (2014). Stage-specific embryonic antigen-4 as a potential therapeutic target in glioblastoma multiforme and other cancers. [Research Support, Non-U.S. Gov't]. Proc Natl Acad Sci U S A, 111(7), 2482-2487. doi: 10.1073/pnas.1400283111

Louvet, C., Labianca, R., Hammel, P., Lledo, G., Zampino, M. G., Andre, T., . . . de Gramont, A. (2005). Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. J Clin Oncol, 23(15), 3509-3516. doi: 10.1200/jco.2005.06.023

Ludwig, D. L., Pereira, D. S., Zhu, Z., Hicklin, D. J., & Bohlen, P. (2003). Monoclonal antibody therapeutics and apoptosis. Oncogene, 22(56), 9097-9106. doi: 10.1038/sj.onc.1207104

Luqman, M., Klabunde, S., Lin, K., Georgakis, G. V., Cherukuri, A., Holash, J., . . . Younes, A. (2008). The antileukemia activity of a human anti-CD40 antagonist antibody, HCD122, on human chronic lymphocytic leukemia cells. Blood, 112(3), 711-720. doi: 10.1182/blood-2007-04-084756

Lynch, T. J., Bondarenko, I., Luft, A., Serwatowski, P., Barlesi, F., Chacko, R., . . . Reck, M. (2012). Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. [Clinical Trial, Phase II

Multicenter Study

Randomized Controlled Trial

Research Support, Non-U.S. Gov't]. J Clin Oncol, 30(17), 2046-2054. doi: 10.1200/JCO.2011.38.4032

Ma, J., Usui, Y., Kezuka, T., Okunuki, Y., Zhang, L., An, X., . . . Goto, H. (2012). Costimulatory molecule expression on human uveal melanoma cells: functional analysis of CD40 and B7-H1. [Research Support, Non-U.S. Gov't]. Exp Eye Res, 96(1), 98-106. doi: 10.1016/j.exer.2011.12.014

Ma, Y., Pannicke, U., Schwarz, K., & Lieber, M. R. (2002). Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. Cell, 108(6), 781-794.

Maccioni, H. J., Giraudo, C. G., & Daniotti, J. L. (2002). Understanding the stepwise synthesis of glycolipids. Neurochem Res, 27(7-8), 629-636.

Macdonald, J. S., Smalley, S. R., Benedetti, J., Hundahl, S. A., Estes, N. C., Stemmermann, G. N., . . . Martenson, J. A. (2001). Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. N Engl J Med, 345(10), 725-730. doi: 10.1056/NEJMoa010187

MacLennan, I. C. (1994). Germinal centers. Annu Rev Immunol, 12, 117-139. doi: 10.1146/annurev.iy.12.040194.001001

Magnusson, S. E., Engstrom, M., Jacob, U., Ulfgren, A. K., & Kleinau, S. (2007). High synovial expression of the inhibitory FcgammaRIIb in rheumatoid arthritis. Arthritis Res Ther, 9(3), R51. doi: 10.1186/ar2206

Mahmoud, S. M., Paish, E. C., Powe, D. G., Macmillan, R. D., Grainge, M. J., Lee, A. H., . . . Green, A. R. (2011). Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. [Research Support, Non-U.S. Gov't]. J Clin Oncol, 29(15), 1949-1955. doi: 10.1200/JCO.2010.30.5037

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol, 146(1), 3-15.

Mangan, N. E., Fallon, R. E., Smith, P., van Rooijen, N., McKenzie, A. N., & Fallon, P. G. (2004). Helminth infection protects mice from anaphylaxis via IL-10-producing B cells. J Immunol, 173(10), 6346-6356.

Mantovani, A., & Allavena, P. (2015). The interaction of anticancer therapies with tumor-associated macrophages. J Exp Med, 212(4), 435-445. doi: 10.1084/jem.20150295

Manz, R. A., Lohning, M., Cassese, G., Thiel, A., & Radbruch, A. (1998). Survival of long-lived plasma cells is independent of antigen. Int Immunol, 10(11), 1703-1711.

Manz, R. A., Thiel, A., & Radbruch, A. (1997). Lifetime of plasma cells in the bone marrow. Nature, 388(6638), 133-134. doi: 10.1038/40540

Mao, S., Gao, C., Lo, C. H., Wirsching, P., Wong, C. H., & Janda, K. D. (1999). Phage-display library selection of high-affinity human single-chain antibodies to tumor-associated carbohydrate antigens sialyl Lewisx and Lewisx. Proc Natl Acad Sci U S A, 96(12), 6953-6958.

Marhaba, R., & Zoller, M. (2004). CD44 in cancer progression: adhesion, migration and growth regulation. J Mol Histol, 35(3), 211-231.

Market, E., & Papavasiliou, F. N. (2003). V(D)J recombination and the evolution of the adaptive immune system. PLoS Biol, 1(1), E16. doi: 10.1371/journal.pbio.0000016

Martin, L. H., Calabi, F., & Milstein, C. (1986). Isolation of CD1 genes: a family of major histocompatibility complex-related differentiation antigens. Proc Natl Acad Sci U S A, 83(23), 9154-9158.

Massard, C., Deutsch, E., & Soria, J. C. (2006). Tumour stem cell-targeted treatment: elimination or differentiation. Ann Oncol, 17(11), 1620-1624. doi: 10.1093/annonc/mdl074

Matsuda, J. L., Mallevaey, T., Scott-Browne, J., & Gapin, L. (2008). CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. Curr Opin Immunol, 20(3), 358-368. doi: 10.1016/j.coi.2008.03.018

Matsusako, T., Muramatsu, H., Shirahama, T., Muramatsu, T., & Ohi, Y. (1991). Expression of a carbohydrate signal, sialyl dimeric Le(x) antigen, is associated with metastatic potential of transitional cell carcinoma of the human urinary bladder. [Research Support, Non-U.S. Gov't]. Biochem Biophys Res Commun, 181(3), 1218-1222.

Mayor, S., Rothberg, K. G., & Maxfield, F. R. (1994). Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. Science, 264(5167), 1948-1951.

McGuire, W. P., Hoskins, W. J., Brady, M. F., Kucera, P. R., Partridge, E. E., Look, K. Y., . . . Davidson, M. (1996). Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med, 334(1), 1-6. doi: 10.1056/nejm199601043340101

McHeyzer-Williams, L. J., & McHeyzer-Williams, M. G. (2005). Antigen-specific memory B cell development. Annu Rev Immunol, 23, 487-513. doi: 10.1146/annurev.immunol.23.021704.115732

Meezan, E., Wu, H. C., Black, P. H., & Robbins, P. W. (1969). Comparative studies on the carbohydrate-containing membrane components of normal and virus-transformed mouse fibroblasts. II. Separation of glycoproteins and glycopeptides by sephadex chromatography. Biochemistry, 8(6), 2518-2524.

Mehta, J., Prabhu, R., Eshpuniyani, P., Kantharia, C., & Supe, A. (2010). Evaluating the efficacy of tumor markers CA 19-9 and CEA to predict operability and survival in pancreatic malignancies. Trop Gastroenterol, 31(3), 190-194.

Meknache, N., Jonsson, F., Laurent, J., Guinnepain, M. T., & Daeron, M. (2009). Human basophils express the glycosylphosphatidylinositol-anchored low-affinity IgG receptor FcgammaRIIIB (CD16B). J Immunol, 182(4), 2542-2550. doi: 10.4049/jimmunol.0801665

Meri, S., Morgan, B. P., Davies, A., Daniels, R. H., Olavesen, M. G., Waldmann, H., & Lachmann, P. J. (1990). Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. Immunology, 71(1), 1-9.

Metelitsa, L. S., Naidenko, O. V., Kant, A., Wu, H. W., Loza, M. J., Perussia, B., . . . Seeger, R. C. (2001). Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. J Immunol, 167(6), 3114-3122.

Michaelsen, T. E., Aase, A., Westby, C., & Sandlie, I. (1990). Enhancement of complement activation and cytolysis of human IgG3 by deletion of hinge exons. Scand J Immunol, 32(5), 517-528.

Miescher, S., Stoeck, M., Qiao, L., Barras, C., Barrelet, L., & von Fliedner, V. (1988). Preferential clonogenic deficit of CD8-positive T-lymphocytes infiltrating human solid tumors. Cancer Res, 48(24 Pt 1), 6992-6998.

Mimura, Y., Ashton, P. R., Takahashi, N., Harvey, D. J., & Jefferis, R. (2007). Contrasting glycosylation profiles between Fab and Fc of a human IgG protein studied by electrospray ionization mass spectrometry. J Immunol Methods, 326(1-2), 116-126. doi: 10.1016/j.jim.2007.07.014

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., & Gianni, L. (2004). Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev, 56(2), 185-229. doi: 10.1124/pr.56.2.6

Mohan, T., Verma, P., & Rao, D. N. (2013). Novel adjuvants & delivery vehicles for vaccines development: a road ahead. [Review]. Indian J Med Res, 138(5), 779-795.

Molina, J. R., Yang, P., Cassivi, S. D., Schild, S. E., & Adjei, A. A. (2008). Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc, 83(5), 584-594. doi: 10.4065/83.5.584

Mond, J. J., Vos, Q., Lees, A., & Snapper, C. M. (1995). T cell independent antigens. Curr Opin Immunol, 7(3), 349-354.

Montecino-Rodriguez, E., & Dorshkind, K. (2012). B-1 B cell development in the fetus and adult. Immunity, 36(1), 13-21. doi: 10.1016/j.immuni.2011.11.017

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol, 19, 683-765. doi: 10.1146/annurev.immunol.19.1.683

Moremen, K. W., Tiemeyer, M., & Nairn, A. V. (2012). Vertebrate protein glycosylation: diversity, synthesis and function. Nat Rev Mol Cell Biol, 13(7), 448-462. doi: 10.1038/nrm3383

Morgan, B. P. (1989). Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochem J, 264(1), 1-14.

Morgan, B. P. (1999). Regulation of the complement membrane attack pathway. Crit Rev Immunol, 19(3), 173-198.

Morrice, N. A., & Powis, S. J. (1998). A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules. Curr Biol, 8(12), 713-716.

Morris Kupchan, S., Komoda, Y., Court, W. A., Thomas, G. J., Smith, R. M., Karim, A., . . . Bryan, R. F. (1972). Tumour inhibitors. LXXIII. Maytansine, a novel antileukemic ansa macrolide from Maytenus ovatus. Journal of the American Chemistry Society, 94(4), 1354-1356.

Mujoo, K., Cheresh, D. A., Yang, H. M., & Reisfeld, R. A. (1987). Disialoganglioside GD2 on human neuroblastoma cells: target antigen for monoclonal antibody-mediated cytolysis and suppression of tumor growth. Cancer Res, 47(4), 1098-1104.

Muller-Eberhard, H. J. (1988). Molecular organization and function of the complement system. Annu Rev Biochem, 57, 321-347. doi: 10.1146/annurev.bi.57.070188.001541

Murai, H., Hara, S., Ikenaka, T., Goto, A., Arai, M., & Murao, S. (1985). Amino acid sequence of protein alpha-amylase inhibitor from Streptomyces griseosporeus YM-25. J Biochem, 97(4), 1129-1133.

Nagae, M., & Yamaguchi, Y. (2012). Function and 3D structure of the N-glycans on glycoproteins. Int J Mol Sci, 13(7), 8398-8429. doi: 10.3390/ijms13078398

Nagata, S., & Golstein, P. (1995). The Fas death factor. Science, 267(5203), 1449-1456.

Nakagoe, T., Fukushima, K., Nanashima, A., Sawai, T., Tsuji, T., Jibiki, M., . . . Arisawa, K. (2000). Expression of Lewis(a), sialyl Lewis(a), Lewis(x) and sialyl Lewis(x) antigens as prognostic factors in patients with colorectal cancer. Can J Gastroenterol, 14(9), 753-760.

Nakayama, T., Watanabe, M., Katsumata, T., Teramoto, T., & Kitajima, M. (1995). Expression of sialyl Lewis(a) as a new prognostic factor for patients with advanced colorectal carcinoma. Cancer, 75(8), 2051-2056.

Naradikian, M. S., Scholz, J. L., Oropallo, M. A., & Cancro, M. P. (2014). Understanding B Cell Biology. 11-35.

Natsume, A., In, M., Takamura, H., Nakagawa, T., Shimizu, Y., Kitajima, K., . . . Niwa, R. (2008). Engineered antibodies of IgG1/IgG3 mixed isotype with enhanced cytotoxic activities. Cancer Res, 68(10), 3863-3872. doi: 10.1158/0008-5472.can-07-6297

Navada, S., Lai, P., Schwartz, A. G., & Kalemkerian, G. P. (2006). Temporal trends in small cell lung cancer: Analysis of the national Surveillance, Epidemiology, and End-Results (SEER) database. Journal of Clinical Oncology, 24(18), 384s-384s.

Nettleship, J. E. (2012). Structural Biology of glycoproteins D. S. Petrescu (Ed.)

Nicholson-Weller, A., March, J. P., Rosen, C. E., Spicer, D. B., & Austen, K. F. (1985). Surface membrane expression by human blood leukocytes and platelets of decay-accelerating factor, a regulatory protein of the complement system. Blood, 65(5), 1237-1244.

Nilsson, O., Brezicka, F. T., Holmgren, J., Sorenson, S., Svennerholm, L., Yngvason, F., & Lindholm, L. (1986). Detection of a ganglioside antigen associated with small cell lung carcinomas using monoclonal antibodies directed against fucosyl-GM1. Cancer Res, 46(3), 1403-1407.

Nimmerjahn, F. (2014). Molecular and cellular pathways of immunoglobulin G activity in vivo. ISRN Immunology, 1-13.

Nimmerjahn, F., & Ravetch, J. V. (2008). Fcgamma receptors as regulators of immune responses. Nat Rev Immunol, 8(1), 34-47. doi: 10.1038/nri2206

Ninomiya, A., Ogasawara, K., Kajino, K., Takada, A., & Kida, H. (2002). Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice. [Research Support, Non-U.S. Gov't]. Vaccine, 20(25-26), 3123-3129.

Niwa, R., Natsume, A., Uehara, A., Wakitani, M., Iida, S., Uchida, K., . . . Shitara, K. (2005). IgG subclass-independent improvement of antibody-dependent cellular cytotoxicity by fucose removal from Asn297-linked oligosaccharides. J Immunol Methods, 306(1-2), 151-160. doi: 10.1016/j.jim.2005.08.009

Noble, P., Spendlove, I., Harding, S., Parsons, T., & Durrant, L. G. (2013). Therapeutic targeting of Lewis(y) and Lewis(b) with a novel monoclonal antibody 692/29. PLoS One, 8(2), e54892. doi: 10.1371/journal.pone.0054892

Nomura, J., Inui, S., Yamasaki, T., Kataoka, S., Maeda, K., Nakanishi, K., & Sakaguchi, N. (1995). Anti-CD40 monoclonal antibody induces the proliferation of murine B cells as a B-cell mitogen through a distinct pathway from receptors for antigens or lipopolysaccharide. [Research Support, Non-U.S. Gov't]. Immunol Lett, 45(3), 195-203.

Norderhaug, L., Brekke, O. H., Bremnes, B., Sandin, R., Aase, A., Michaelsen, T. E., & Sandlie, I. (1991). Chimeric mouse human IgG3 antibodies with an IgG4-like hinge region induce complement-mediated lysis more efficiently than IgG3 with normal hinge. Eur J Immunol, 21(10), 2379-2384. doi: 10.1002/eji.1830211013

Nose, M., Katoh, M., Okada, N., Kyogoku, M., & Okada, H. (1990). Tissue distribution of HRF20, a novel factor preventing the membrane attack of homologous complement, and its predominant expression on endothelial cells in vivo. Immunology, 70(2), 145-149.

Noto, Z., Yoshida, T., Okabe, M., Koike, C., Fathy, M., Tsuno, H., . . . Nikaido, T. (2013). CD44 and SSEA-4 positive cells in an oral cancer cell line HSC-4 possess cancer stem-like cell characteristics. Oral Oncol, 49(8), 787-795. doi: 10.1016/j.oraloncology.2013.04.012

Nudelman, E., Hakomori, S., Kannagi, R., Levery, S., Yeh, M. Y., Hellstrom, K. E., & Hellstrom, I. (1982). Characterization of a human melanoma-associated ganglioside antigen defined by a monoclonal antibody, 4.2. [Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. J Biol Chem, 257(21), 12752-12756.

Obukhanych, T. V., & Nussenzweig, M. C. (2006). T-independent type II immune responses generate memory B cells. J Exp Med, 203(2), 305-310. doi: 10.1084/jem.20052036

Okayama, Y., Kirshenbaum, A. S., & Metcalfe, D. D. (2000). Expression of a functional high-affinity IgG receptor, Fc gamma RI, on human mast cells: Up-regulation by IFN-gamma. J Immunol, 164(8), 4332-4339.

Okazaki, A., Shoji-Hosaka, E., Nakamura, K., Wakitani, M., Uchida, K., Kakita, S., . . . Shitara, K. (2004). Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcgammaRIIIa. J Mol Biol, 336(5), 1239-1249. doi: 10.1016/j.jmb.2004.01.007

Oldenburg, K. R., Loganathan, D., Goldstein, I. J., Schultz, P. G., & Gallop, M. A. (1992). Peptide ligands for a sugar-binding protein isolated from a random peptide library. [Comparative Study]. Proc Natl Acad Sci U S A, 89(12), 5393-5397.

Ortegren, U., Karlsson, M., Blazic, N., Blomqvist, M., Nystrom, F. H., Gustavsson, J., . . . Stralfors, P. (2004). Lipids and glycosphingolipids in caveolae and surrounding plasma membrane of primary rat adipocytes. Eur J Biochem, 271(10), 2028-2036. doi: 10.1111/j.1432-1033.2004.04117.x

Orth, J. D., Krueger, E. W., Weller, S. G., & McNiven, M. A. (2006). A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. Cancer Res, 66(7), 3603-3610. doi: 10.1158/0008-5472.can-05-2916

Osborne, C. K., & Schiff, R. (2011). Mechanisms of endocrine resistance in breast cancer. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't

Review]. Annu Rev Med, 62, 233-247. doi: 10.1146/annurev-med-070909-182917

Ostman, A., & Augsten, M. (2009). Cancer-associated fibroblasts and tumor growth--bystanders turning into key players. [Review]. Curr Opin Genet Dev, 19(1), 67-73. doi: 10.1016/j.gde.2009.01.003

Padler-Karavani, V. (2014). Aiming at the sweet side of cancer: aberrant glycosylation as possible target for personalized-medicine. Cancer Lett, 352(1), 102-112. doi: 10.1016/j.canlet.2013.10.005

Panka, D. J. (1997). Glycosylation is influential in murine IgG3 self-association. Mol Immunol, 34(8-9), 593-598.

Panowksi, S., Bhakta, S., Raab, H., Polakis, P., & Junutula, J. R. (2014). Site-specific antibody drug conjugates for cancer therapy. MAbs, 6(1), 34-45. doi: 10.4161/mabs.27022

Parker, D. C., Wadsworth, D. C., & Schneider, G. B. (1980). Activation of murine B lymphocytes by anti-immunoglobulin is an inductive signal leading to immunoglobulin secretion. J Exp Med, 152(1), 138-150.

Parton, R. G., & del Pozo, M. A. (2013). Caveolae as plasma membrane sensors, protectors and organizers. Nat Rev Mol Cell Biol, 14(2), 98-112. doi: 10.1038/nrm3512

Pelayo, R., Welner, R. S., Nagai, Y., & Kincade, P. W. (2006). Life before the pre-B cell receptor checkpoint: specification and commitment of primitive lymphoid progenitors in adult bone marrow. Semin Immunol, 18(1), 2-11. doi: 10.1016/j.smim.2005.10.005

Perez-Andres, M., Paiva, B., Nieto, W. G., Caraux, A., Schmitz, A., Almeida, J., . . . Orfao, A. (2010). Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. Cytometry B Clin Cytom, 78 Suppl 1, S47-60. doi: 10.1002/cyto.b.20547

Perez-Soler, R., Chachoua, A., Hammond, L. A., Rowinsky, E. K., Huberman, M., Karp, D., . . . Bonomi, P. (2004). Determinants of tumor response and survival with erlotinib in patients with non--small-cell lung cancer. J Clin Oncol, 22(16), 3238-3247. doi: 10.1200/jco.2004.11.057

Pescovitz, M. D. (2006). Rituximab, an anti-cd20 monoclonal antibody: history and mechanism of action. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't

Review]. Am J Transplant, 6(5 Pt 1), 859-866. doi: 10.1111/j.1600-6143.2006.01288.x

Petersdorf, S., Kopecky, K., Stuart, R. K., Larson, R. A., Nevill, T. J., Stenke, L., . . . Appelbaum, F. R. (2009). Preliminary Results of Southwest Oncology Group Study S0106: An International Intergroup Phase 3 Randomized Trial Comparing the Addition of Gemtuzumab Ozogamicin to Standard Induction Therapy Versus Standard Induction Therapy Followed by a Second Randomization to Post-Consolidation Gemtuzumab Ozogamicin Versus No Additional Therapy for Previously Untreated Acute Myeloid Leukemia. Blood, 114(22), 326-327.

Petroff, M. G., Chen, L., Phillips, T. A., & Hunt, J. S. (2002). B7 family molecules: novel immunomodulators at the maternal-fetal interface. [Research Support, U.S. Gov't, P.H.S.

Review]. Placenta, 23 Suppl A, S95-101. doi: 10.1053/plac.2002.0813

Pillai, S., & Baltimore, D. (1987). Formation of disulphide-linked mu 2 omega 2 tetramers in pre-B cells by the 18K omega-immunoglobulin light chain. Nature, 329(6135), 172-174. doi: 10.1038/329172a0

Pillai, S., Netravali, I. A., Cariappa, A., & Mattoo, H. (2012). Siglecs and immune regulation. Annu Rev Immunol, 30, 357-392. doi: 10.1146/annurev-immunol-020711-075018

Pitto, M., Brunner, J., Ferraretto, A., Ravasi, D., Palestini, P., & Masserini, M. (2000). Use of a photoactivable GM1 ganglioside analogue to assess lipid distribution in caveolae bilayer. Glycoconj J, 17(3 -4), 215-222.

Poplin, E., Feng, Y., Berlin, J., Rothenberg, M. L., Hochster, H., Mitchell, E., . . . Benson, A. B., 3rd. (2009). Phase III, randomized study of gemcitabine and oxaliplatin versus gemcitabine (fixed-dose rate infusion) compared with gemcitabine (30-minute infusion) in patients with pancreatic carcinoma E6201: a trial of the Eastern Cooperative Oncology Group. J Clin Oncol, 27(23), 3778-3785. doi: 10.1200/jco.2008.20.9007

Porcelli, S., Morita, C. T., & Brenner, M. B. (1992). CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen. Nature, 360(6404), 593-597. doi: 10.1038/360593a0

Porcelli, S. A. (1995). The CD1 family: a third lineage of antigen-presenting molecules. Adv Immunol, 59, 1-98.

Porcelli, S. A., & Modlin, R. L. (1999). The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. Annu Rev Immunol, 17, 297-329. doi: 10.1146/annurev.immunol.17.1.297

Post, T. W., Arce, M. A., Liszewski, M. K., Thompson, E. S., Atkinson, J. P., & Lublin, D. M. (1990). Structure of the gene for human complement protein decay accelerating factor. J Immunol, 144(2), 740-744.

Purves, W. K., Orians, G. H., & Heller, H. C. (1995). Life, the science of biology (4th ed.). Sunderland, Mass.

Salt Lake City, Utah: Sinauer Associates ;

W.H. Freeman.

Qian, B. Z., & Pollard, J. W. (2010). Macrophage diversity enhances tumor progression and metastasis. Cell, 141(1), 39-51. doi: 10.1016/j.cell.2010.03.014

Qin, J., Han, B., & Pang, J. (1997). [The relationship between TIL from human primary hepatic carcinoma and prognosis]. Zhonghua Yi Xue Za Zhi, 77(3), 167-170.

Quatromoni, J. G., & Eruslanov, E. (2012). Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. Am J Transl Res, 4(4), 376-389.

Ra, C., Jouvin, M. H., Blank, U., & Kinet, J. P. (1989). A macrophage Fc gamma receptor and the mast cell receptor for IgE share an identical subunit. Nature, 341(6244), 752-754. doi: 10.1038/341752a0

Rabu, C., McIntosh, R., Jurasova, Z., & Durrant, L. (2012). Glycans as targets for therapeutic antitumor antibodies. Future Oncol, 8(8), 943-960. doi: 10.2217/fon.12.88

Radic, M. Z., & Zouali, M. (1996). Receptor editing, immune diversification, and self-tolerance. Immunity, 5(6), 505-511.

Raja, F. A., Chopra, N., & Ledermann, J. A. (2012). Optimal first-line treatment in ovarian cancer. Ann Oncol, 23 Suppl 10, x118-127. doi: 10.1093/annonc/mds315

Raju, T. S. (2008). Terminal sugars of Fc glycans influence antibody effector functions of IgGs. [Review]. Curr Opin Immunol, 20(4), 471-478. doi: 10.1016/j.coi.2008.06.007

Rakha, E. A., El-Sheikh, S. E., Kandil, M. A., El-Sayed, M. E., Green, A. R., & Ellis, I. O. (2008). Expression of BRCA1 protein in breast cancer and its prognostic significance. Hum Pathol, 39(6), 857-865. doi: 10.1016/j.humpath.2007.10.011

Ramalingam, S., & Belani, C. (2008). Systemic chemotherapy for advanced non-small cell lung cancer: recent advances and future directions. Oncologist, 13 Suppl 1, 5-13. doi: 10.1634/theoncologist.13-S1-5

Ramsden, D. A., & Gellert, M. (1998). Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double-strand breaks. EMBO J, 17(2), 609-614. doi: 10.1093/emboj/17.2.609

Ravandi, F. (2011). Gemtuzumab ozogamicin: one size does not fit all--the case for personalized therapy. J Clin Oncol, 29(4), 349-351. doi: 10.1200/jco.2010.32.2693

Ravetch, J. V., & Kinet, J. P. (1991). Fc receptors. Annu Rev Immunol, 9, 457-492. doi: 10.1146/annurev.iy.09.040191.002325

Ravetch, J. V., & Lanier, L. L. (2000). Immune inhibitory receptors. Science, 290(5489), 84-89.

Ravn, V., & Dabelsteen, E. (2000). Tissue distribution of histo-blood group antigens. APMIS, 108(1), 1-28.

Reese, Z., Straubhar, A., Pal, S. K., & Agarwal, N. (2015). Ipilimumab in the treatment of prostate cancer. Future Oncol, 11(1), 27-37. doi: 10.2217/fon.14.196

Reichert, J. M. (2009). Probabilities of success for antibody therapeutics. MAbs, 1(4), 387-389.

Reichert, J. M. (2010). Antibodies to watch in 2010. MAbs, 2(1), 84-100.

Reichert, J. M. (2011). Antibody-based therapeutics to watch in 2011. MAbs, 3(1), 76-99.

Reth, M. (1989). Antigen receptor tail clue. Nature, 338(6214), 383-384. doi: 10.1038/338383b0

Ricart, A. D., & Tolcher, A. W. (2007). Technology insight: cytotoxic drug immunoconjugates for cancer therapy. Nat Clin Pract Oncol, 4(4), 245-255. doi: 10.1038/ncponc0774

Richter, T., Floetenmeyer, M., Ferguson, C., Galea, J., Goh, J., Lindsay, M. R., . . . Parton, R. G. (2008). High-resolution 3D quantitative analysis of caveolar ultrastructure and caveola-cytoskeleton interactions. Traffic, 9(6), 893-909. doi: 10.1111/j.1600-0854.2008.00733.x

Ritchie, M., Tchistiakova, L., & Scott, N. (2013). Implications of receptor-mediated endocytosis and intracellular trafficking dynamics in the development of antibody drug conjugates. MAbs, 5(1), 13-21. doi: 10.4161/mabs.22854

Rizvi, N. A., Mazieres, J., Planchard, D., Stinchcombe, T. E., Dy, G. K., Antonia, S. J., . . . Ramalingam, S. S. (2015). Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial. [Clinical Trial, Phase II

Multicenter Study

Research Support, Non-U.S. Gov't]. Lancet Oncol, 16(3), 257-265. doi: 10.1016/S1470-2045(15)70054-9

Robert, C., Ribas, A., Wolchok, J. D., Hodi, F. S., Hamid, O., Kefford, R., . . . Daud, A. (2014). Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. [Clinical Trial, Phase I

Comparative Study

Multicenter Study

Randomized Controlled Trial

Research Support, Non-U.S. Gov't]. Lancet, 384(9948), 1109-1117. doi: 10.1016/S0140-6736(14)60958-2

Robert, C., Schachter, J., Long, G. V., Arance, A., Grob, J. J., Mortier, L., . . . Ribas, A. (2015). Pembrolizumab versus Ipilimumab in Advanced Melanoma. [Clinical Trial, Phase III

Comparative Study

Multicenter Study

Randomized Controlled Trial

Research Support, Non-U.S. Gov't]. N Engl J Med, 372(26), 2521-2532. doi: 10.1056/NEJMoa1503093

Robert, C., Thomas, L., Bondarenko, I., O'Day, S., Weber, J., Garbe, C., . . . Wolchok, J. D. (2011). Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. [Clinical Trial, Phase III

Multicenter Study

Randomized Controlled Trial

Research Support, Non-U.S. Gov't]. N Engl J Med, 364(26), 2517-2526. doi: 10.1056/NEJMoa1104621

Rodon, J., Garrison, M., Hammond, L. A., de Bono, J., Smith, L., Forero, L., . . . Tolcher, A. W. (2008). Cantuzumab mertansine in a three-times a week schedule: a phase I and pharmacokinetic study. [Clinical Trial, Phase I]. Cancer Chemother Pharmacol, 62(5), 911-919. doi: 10.1007/s00280-007-0672-8

Romano, A., Conticello, C., Cavalli, M., Vetro, C., Di Raimondo, C., Di Martina, V., . . . Di Raimondo, F. (2014). Salvage therapy of multiple myeloma: the new generation drugs. [Research Support, Non-U.S. Gov't

Review]. Biomed Res Int, 2014, 456037. doi: 10.1155/2014/456037

Ronnberg, B., Fekadu, M., & Morein, B. (1995). Adjuvant activity of non-toxic Quillaja saponaria Molina components for use in ISCOM matrix. [Comparative Study

Research Support, Non-U.S. Gov't]. Vaccine, 13(14), 1375-1382.

Royal, R. E., Levy, C., Turner, K., Mathur, A., Hughes, M., Kammula, U. S., . . . Rosenberg, S. A. (2010). Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. J Immunother, 33(8), 828-833. doi: 10.1097/CJI.0b013e3181eec14c

Rudnick, S. I., Lou, J., Shaller, C. C., Tang, Y., Klein-Szanto, A. J., Weiner, L. M., . . . Adams, G. P. (2011). Influence of affinity and antigen internalization on the uptake and penetration of Anti-HER2 antibodies in solid tumors. Cancer Res, 71(6), 2250-2259. doi: 10.1158/0008-5472.can-10-2277

Rutkowski, M. J., Sughrue, M. E., Kane, A. J., Mills, S. A., & Parsa, A. T. (2010). Cancer and the complement cascade. Mol Cancer Res, 8(11), 1453-1465. doi: 10.1158/1541-7786.mcr-10-0225

Ryan, D. P., Hong, T. S., & Bardeesy, N. (2014). Pancreatic adenocarcinoma. N Engl J Med, 371(11), 1039-1049. doi: 10.1056/NEJMra1404198

Safi, F., Roscher, R., Bittner, R., Schenkluhn, B., Dopfer, H. P., & Beger, H. G. (1987). High sensitivity and specificity of CA 19-9 for pancreatic carcinoma in comparison to chronic pancreatitis. Serological and immunohistochemical findings. [Comparative Study]. Pancreas, 2(4), 398-403.

Sagiv, E., Memeo, L., Karin, A., Kazanov, D., Jacob-Hirsch, J., Mansukhani, M., . . . Arber, N. (2006). CD24 is a new oncogene, early at the multistep process of colorectal cancer carcinogenesis. Gastroenterology, 131(2), 630-639. doi: 10.1053/j.gastro.2006.04.028

Sagiv, E., Starr, A., Rozovski, U., Khosravi, R., Altevogt, P., Wang, T., & Arber, N. (2008). Targeting CD24 for treatment of colorectal and pancreatic cancer by monoclonal antibodies or small interfering RNA. Cancer Res, 68(8), 2803-2812. doi: 10.1158/0008-5472.can-07-6463

Saito, S., Orikasa, S., Satoh, M., Ohyama, C., Ito, A., & Takahashi, T. (1997). Expression of globo-series gangliosides in human renal cell carcinoma. Jpn J Cancer Res, 88(7), 652-659.

Sakamoto, J., Furukawa, K., Cordon-Cardo, C., Yin, B. W., Rettig, W. J., Oettgen, H. F., . . . Lloyd, K. O. (1986). Expression of Lewisa, Lewisb, X, and Y blood group antigens in human colonic tumors and normal tissue and in human tumor-derived cell lines. Cancer Res, 46(3), 1553-1561.

Sakamoto, S., Watanabe, T., Tokumaru, T., Takagi, H., Nakazato, H., & Lloyd, K. O. (1989). Expression of Lewisa, Lewisb, Lewisx, Lewisy, siayl-Lewisa, and sialyl-Lewisx blood group antigens in human gastric carcinoma and in normal gastric tissue. Cancer Res, 49(3), 745-752.

Salazar, G., & Gonzalez, A. (2002). Novel mechanism for regulation of epidermal growth factor receptor endocytosis revealed by protein kinase A inhibition. Mol Biol Cell, 13(5), 1677-1693. doi: 10.1091/mbc.01-08-0403

Saldova, R., Fan, Y., Fitzpatrick, J. M., Watson, R. W., & Rudd, P. M. (2011). Core fucosylation and alpha2-3 sialylation in serum N-glycome is significantly increased in prostate cancer comparing to benign prostate hyperplasia. Glycobiology, 21(2), 195-205. doi: 10.1093/glycob/cwq147

Samelis, G. F., Ekmektzoglou, K. A., Tsiakou, A., & Konstadoulakis, M. (2011). The continuation of bevacizumab following disease progression in patients with metastatic colorectal cancer offers a survival benefit. Hepatogastroenterology, 58(112), 1968-1971. doi: 10.5754/hge11179

Sandler, A. B., Gray, R., Brahmer, J., Dowlati, A., Schiller, J. H., Perry, M. C., & Johnson, D. H. (2005). Randomised phase II/III trial of paclitaxel (P) plus carboplatin © with or without bevacizumab (NSC#704865) in patients with advanced non-squamous, non-small cell lung cancer (NSCLC): an Eastern Cooperative Oncology Group (ECOG) Trial E4599 Journal of Clinical of Oncology 23(16S).

Santana-Davila, R., & Perez, E. A. (2010). Treatment options for patients with triple-negative breast cancer. Journal of Hematology & Oncology, 3. doi: Artn 42

10.1186/1756-87223-42

Sargiacomo, M., Sudol, M., Tang, Z., & Lisanti, M. P. (1993). Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J Cell Biol, 122(4), 789-807.

Sawada, R., Lowe, J. B., & Fukuda, M. (1993). E-selectin-dependent adhesion efficiency of colonic carcinoma cells is increased by genetic manipulation of their cell surface lysosomal membrane glycoprotein-1 expression levels. [Research Support, U.S. Gov't, P.H.S.]. J Biol Chem, 268(17), 12675-12681.

Sawada, R., Sun, S. M., Wu, X., Hong, F., Ragupathi, G., Livingston, P. O., & Scholz, W. W. (2011). Human monoclonal antibodies to sialyl-Lewis (CA19.9) with potent CDC, ADCC, and antitumor activity. Clin Cancer Res, 17(5), 1024-1032. doi: 10.1158/1078-0432.ccr-10-2640

Schengrund, C. L. (1990). The role(s) of gangliosides in neural differentiation and repair: a perspective. Brain Res Bull, 24(1), 131-141.

Schirrmann, T., Meyer, T., Schutte, M., Frenzel, A., & Hust, M. (2011). Phage display for the generation of antibodies for proteome research, diagnostics and therapy. Molecules, 16(1), 412-426. doi: 10.3390/molecules16010412

Schnaar, R. L. (2004). Glycolipid-mediated cell-cell recognition in inflammation and nerve regeneration. Arch Biochem Biophys, 426(2), 163-172. doi: 10.1016/j.abb.2004.02.019

Schneider, F., Kemmner, W., Haensch, W., Franke, G., Gretschel, S., Karsten, U., & Schlag, P. M. (2001). Overexpression of sialyltransferase CMP-sialic acid:Galbeta1,3GalNAc-R alpha6-Sialyltransferase is related to poor patient survival in human colorectal carcinomas. Cancer Res, 61(11), 4605-4611.

Schofield, D. J., Pope, A. R., Clementel, V., Buckell, J., Chapple, S., Clarke, K. F., . . . McCafferty, J. (2007). Application of phage display to high throughput antibody generation and characterization. [Research Support, Non-U.S. Gov't]. Genome Biol, 8(11), R254. doi: 10.1186/gb-2007-8-11-r254

Schoonbroodt, S., Steukers, M., Viswanathan, M., Frans, N., Timmermans, M., Wehnert, A., . . . Hoet, R. M. (2008). Engineering antibody heavy chain CDR3 to create a phage display Fab library rich in antibodies that bind charged carbohydrates. [Validation Studies]. J Immunol, 181(9), 6213-6221.

Schreiber, G. J., Hellstrom, K. E., & Hellstrom, I. (1992). An unmodified anticarcinoma antibody, BR96, localizes to and inhibits the outgrowth of human tumors in nude mice. Cancer Res, 52(12), 3262-3266.

Schroeder, H. W., Jr., & Cavacini, L. (2010). Structure and function of immunoglobulins. J Allergy Clin Immunol, 125(2 Suppl 2), S41-52. doi: 10.1016/j.jaci.2009.09.046

Schumaker, V. N., Calcott, M. A., Spiegelberg, H. L., & Muller-Eberhard, H. J. (1976). Ultracentifuge studies of the binding of IgG of different subclasses to the Clq subunit of the first component of complement. Biochemistry, 15(23), 5175-5181.

Schwendener, R. A. (2014). Liposomes as vaccine delivery systems: a review of the recent advances. [Review]. Ther Adv Vaccines, 2(6), 159-182. doi: 10.1177/2051013614541440

Scott, A. M., Wolchok, J. D., & Old, L. J. (2012). Antibody therapy of cancer. Nat Rev Cancer, 12(4), 278-287. doi: 10.1038/nrc3236

Scott, J. K., Loganathan, D., Easley, R. B., Gong, X., & Goldstein, I. J. (1992). A family of concanavalin A-binding peptides from a hexapeptide epitope library. [Comparative Study

Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. Proc Natl Acad Sci U S A, 89(12), 5398-5402.

Sela-Culang, I., Kunik, V., & Ofran, Y. (2013). The structural basis of antibody-antigen recognition. Front Immunol, 4, 302. doi: 10.3389/fimmu.2013.00302

Selenko-Gebauer, N., Majdic, O., Szekeres, A., Hofler, G., Guthann, E., Korthauer, U., . . . Stockl, J. (2003). B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy. [Research Support, Non-U.S. Gov't]. J Immunol, 170(7), 3637-3644.

Sewell, R., Backstrom, M., Dalziel, M., Gschmeissner, S., Karlsson, H., Noll, T., . . . Taylor-Papadimitriou, J. (2006). The ST6GalNAc-I sialyltransferase localizes throughout the Golgi

and is responsible for the synthesis of the tumor-associated sialyl-Tn O-glycan in human breast cancer. J Biol Chem, 281(6), 3586-3594. doi: 10.1074/jbc.M511826200

Shah, J. A., Darrah, P. A., Ambrozak, D. R., Turon, T. N., Mendez, S., Kirman, J., . . . Seder, R. A. (2003). Dendritic cells are responsible for the capacity of CpG oligodeoxynucleotides to act as an adjuvant for protective vaccine immunity against Leishmania major in mice. J Exp Med, 198(2), 281-291. doi: 10.1084/jem.20030645

Sher, T., Dy, G. K., & Adjei, A. A. (2008). Small cell lung cancer. Mayo Clin Proc, 83(3), 355-367. doi: 10.4065/83.3.355

Shevinsky, L. H., Knowles, B. B., Damjanov, I., & Solter, D. (1982). Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. Cell, 30(3), 697-705.

Shields, R. L., Lai, J., Keck, R., O'Connell, L. Y., Hong, K., Meng, Y. G., . . . Presta, L. G. (2002). Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem, 277(30), 26733-26740. doi: 10.1074/jbc.M202069200

Shimono, R., Mori, M., Akazawa, K., Adachi, Y., & Sgimachi, K. (1994). Immunohistochemical expression of carbohydrate antigen 19-9 in colorectal carcinoma. Am J Gastroenterol, 89(1), 101-105.

Shogomori, H., & Futerman, A. H. (2001). Cholera toxin is found in detergent-insoluble rafts/domains at the cell surface of hippocampal neurons but is internalized via a raft-independent mechanism. J Biol Chem, 276(12), 9182-9188. doi: 10.1074/jbc.M009414200

Simons, K., & Ikonen, E. (1997). Functional rafts in cell membranes. Nature, 387(6633), 569-572. doi: 10.1038/42408

Singh, R. D., Puri, V., Valiyaveettil, J. T., Marks, D. L., Bittman, R., & Pagano, R. E. (2003). Selective caveolin-1-dependent endocytosis of glycosphingolipids. Mol Biol Cell, 14(8), 3254-3265. doi: 10.1091/mbc.E02-12-0809

Siskind, G. W., & Benacerraf, B. (1969). Cell selection by antigen in the immune response. Adv Immunol, 10, 1-50.

Skrzypski, M., Dziadziuszko, R., Jassem, E., Szymanowska-Narloch, A., Gulida, G., Rzepko, R., . . . Jassem, J. (2013). Main histologic types of non-small-cell lung cancer differ in expression of prognosis-related genes. Clin Lung Cancer, 14(6), 666-673 e662. doi: 10.1016/j.cllc.2013.04.010

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., & McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science, 235(4785), 177-182.

Slifka, M. K., Antia, R., Whitmire, J. K., & Ahmed, R. (1998). Humoral immunity due to long-lived plasma cells. Immunity, 8(3), 363-372.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science, 228(4705), 1315-1317.

Smith, K. G., & Clatworthy, M. R. (2010). FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications. Nat Rev Immunol, 10(5), 328-343. doi: 10.1038/nri2762

Soejima, M., & Koda, Y. (2005). Molecular mechanisms of Lewis antigen expression. Leg Med (Tokyo), 7(4), 266-269. doi: 10.1016/j.legalmed.2004.12.003

Solter, D., & Knowles, B. B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc Natl Acad Sci U S A, 75(11), 5565-5569.

Sotomayor, E. M., Borrello, I., Tubb, E., Rattis, F. M., Bien, H., Lu, Z., . . . Levitsky, H. I. (1999). Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through in vivo ligation of CD40. [Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. Nat Med, 5(7), 780-787. doi: 10.1038/10503

Spiro, R. G. (2002). Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology, 12(4), 43R-56R.

Stahlhut, M., & van Deurs, B. (2000). Identification of filamin as a novel ligand for caveolin-1: evidence for the organization of caveolin-1-associated membrane domains by the actin cytoskeleton. Mol Biol Cell, 11(1), 325-337.

Stills, H. F., Jr. (2005). Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J, 46(3), 280-293.

Stober, D., Jomantaite, I., Schirmbeck, R., & Reimann, J. (2003). NKT cells provide help for dendritic cell-dependent priming of MHC class I-restricted CD8+ T cells in vivo. J Immunol, 170(5), 2540-2548.

Storr, S. J., Zaitoun, A. M., Arora, A., Durrant, L. G., Lobo, D. N., Madhusudan, S., & Martin, S. G. (2012). Calpain system protein expression in carcinomas of the pancreas, bile duct and ampulla. [Research Support, Non-U.S. Gov't]. BMC Cancer, 12, 511. doi: 10.1186/1471-2407-12-511

Strohl, W. R., & Knight, D. M. (2009). Discovery and development of biopharmaceuticals: current issues. [Editorial]. Curr Opin Biotechnol, 20(6), 668-672. doi: 10.1016/j.copbio.2009.10.012

Strome, S. E., Sausville, E. A., & Mann, D. (2007). A mechanistic perspective of monoclonal antibodies in cancer therapy beyond target-related effects. Oncologist, 12(9), 1084-1095. doi: 10.1634/theoncologist.12-9-1084

Stroud, M. R., Levery, S. B., Maartensson, S., Salyan, M. E. K., Clausen, H., & Hakomori, S. (1994). Human tumour-associated Le(a)-Le(x) hybrid carbohydrate antigen IV3 (Galbeta 1->3[Fuc alpha 1->4]GlcNAc)III3FucnLc4 defined by monoclonal antibody 43-9F: enzymatic synthesis, structural characterisation, and comparative reactivity with various antibodies. Biochemistry, 33(35), 10672-10680.

Suzuki, Y., Haraguchi, N., Takahashi, H., Uemura, M., Nishimura, J., Hata, T., . . . Yamamoto, H. (2013). SSEA-3 as a novel amplifying cancer cell surface marker in colorectal cancers. Int J Oncol, 42(1), 161-167. doi: 10.3892/ijo.2012.1713

Taccioli, G. E., Rathbun, G., Oltz, E., Stamato, T., Jeggo, P. A., & Alt, F. W. (1993). Impairment of V(D)J recombination in double-strand break repair mutants. Science, 260(5105), 207-210.

Tan, H. L., Fong, W. J., Lee, E. H., Yap, M., & Choo, A. (2009). mAb 84, a cytotoxic antibody that kills undifferentiated human embryonic stem cells via oncosis. Stem Cells, 27(8), 1792-1801. doi: 10.1002/stem.109

Tan, L. K., Shopes, R. J., Oi, V. T., & Morrison, S. L. (1990). Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins. Proc Natl Acad Sci U S A, 87(1), 162-166.

Tao, M. H., Canfield, S. M., & Morrison, S. L. (1991). The differential ability of human IgG1 and IgG4 to activate complement is determined by the COOH-terminal sequence of the CH2 domain. J Exp Med, 173(4), 1025-1028.

Tao, M. H., & Morrison, S. L. (1989). Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J Immunol, 143(8), 2595-2601.

Teicher, B. A., & Chari, R. V. (2011). Antibody conjugate therapeutics: challenges and potential. Clin Cancer Res, 17(20), 6389-6397. doi: 10.1158/1078-0432.ccr-11-1417

Teillaud, J.-L. (2012). Antibody-dependent cellular cytotoxicity (ADCC). John Willey & Sons, Ltd, 1-8.

Thorbecke, G. J., Asofsky, R. M., Hochwald, G. M., & Siskind, G. W. (1962). Gamma globulin and antibody formation in vitro. III. Induction of secondary response at different intervals after the primary; the role of secondary nodules in the preparation for the secondary response. J Exp Med, 116, 295-310.

Thurman, J. M., & Holers, V. M. (2006). The central role of the alternative complement pathway in human disease. J Immunol, 176(3), 1305-1310.

Tippett, P., Andrews, P. W., Knowles, B. B., Solter, D., & Goodfellow, P. N. (1986). Red cell antigens P (globoside) and Luke: identification by monoclonal antibodies defining the murine stage-specific embryonic antigens -3 and -4 (SSEA-3 and SSEA-4). [Research Support, U.S. Gov't, P.H.S.]. Vox Sang, 51(1), 53-56.

Tolcher, A. W., Ochoa, L., Hammond, L. A., Patnaik, A., Edwards, T., Takimoto, C., . . . Rowinsky, E. K. (2003). Cantuzumab mertansine, a maytansinoid immunoconjugate directed to the CanAg antigen: a phase I, pharmacokinetic, and biologic correlative study. [Clinical Trial

Clinical Trial, Phase I

Comparative Study

Research Support, Non-U.S. Gov't]. J Clin Oncol, 21(2), 211-222.

Tolcher, A. W., Sugarman, S., Gelmon, K. A., Cohen, R., Saleh, M., Isaacs, C., . . . Slichenmyer, W. (1999). Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer. J Clin Oncol, 17(2), 478-484.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature, 302(5909), 575-581.

Topalian, S. L., Sznol, M., McDermott, D. F., Kluger, H. M., Carvajal, R. D., Sharfman, W. H., . . . Hodi, F. S. (2014). Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. [Clinical Trial

Research Support, Non-U.S. Gov't]. J Clin Oncol, 32(10), 1020-1030. doi: 10.1200/JCO.2013.53.0105

Torgersen, M. L., Skretting, G., van Deurs, B., & Sandvig, K. (2001). Internalization of cholera toxin by different endocytic mechanisms. J Cell Sci, 114(Pt 20), 3737-3747.

Tozawa, K., Okamoto, T., Kawai, N., Hashimoto, Y., Hayashi, Y., & Kohri, K. (2005). Positive correlation between sialyl Lewis X expression and pathologic findings in renal cell carcinoma. Kidney Int, 67(4), 1391-1396. doi: 10.1111/j.1523-1755.2005.00216.x

Tran, D., Carpentier, J. L., Sawano, F., Gorden, P., & Orci, L. (1987). Ligands Internalized through Coated or Noncoated Invaginations Follow a Common Intracellular Pathway. Proceedings of the National Academy of Sciences of the United States of America, 84(22), 7957-7961. doi: DOI 10.1073/pnas.84.22.7957

Tsuboi, I., Tanaka, H., Nakao, M., Shichijo, S., & Itoh, K. (1995). Nonsteroidal anti-inflammatory drugs differentially regulate cytokine production in human lymphocytes: up-regulation of TNF, IFN-gamma and IL-2, in contrast to down-regulation of IL-6 production. Cytokine, 7(4), 372-379. doi: 10.1006/cyto.1995.0047

Tsuchida, A., Okajima, T., Furukawa, K., Ando, T., Ishida, H., Yoshida, A., . . . Furukawa, K. (2003). Synthesis of disialyl Lewis a (Le(a)) structure in colon cancer cell lines by a sialyltransferase, ST6GalNAc VI, responsible for the synthesis of alpha-series gangliosides. J Biol Chem, 278(25), 22787-22794. doi: 10.1074/jbc.M211034200

Tsuji, M. (2006). Glycolipids and phospholipids as natural CD1d-binding NKT cell ligands. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't

Review]. Cell Mol Life Sci, 63(16), 1889-1898. doi: 10.1007/s00018-006-6073-z

Tsushima, F., Yao, S., Shin, T., Flies, A., Flies, S., Xu, H., . . . Chen, L. (2007). Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy. [Research Support, N.I.H., Extramural]. Blood, 110(1), 180-185. doi: 10.1182/blood-2006-11-060087

Tuccillo, F. M., de Laurentiis, A., Palmieri, C., Fiume, G., Bonelli, P., Borrelli, A., . . . Scala, G. (2014). Aberrant glycosylation as biomarker for cancer: focus on CD43. Biomed Res Int, 2014, 742831. doi: 10.1155/2014/742831

Twigg, H. L., 3rd. (2005). Humoral immune defense (antibodies): recent advances. Proc Am Thorac Soc, 2(5), 417-421. doi: 10.1513/pats.200508-089JS

Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H., & Bailey, J. E. (1999). Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. Nat Biotechnol, 17(2), 176-180. doi: 10.1038/6179

Van Cutsem, E., Kohne, C. H., Hitre, E., Zaluski, J., Chang Chien, C. R., Makhson, A., . . . Rougier, P. (2009). Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med, 360(14), 1408-1417. doi: 10.1056/NEJMoa0805019

van der Heijden, J., Breunis, W. B., Geissler, J., de Boer, M., van den Berg, T. K., & Kuijpers, T. W. (2012). Phenotypic variation in IgG receptors by nonclassical FCGR2C alleles. J Immunol, 188(3), 1318-1324. doi: 10.4049/jimmunol.1003945

van Gent, D. C., McBlane, J. F., Ramsden, D. A., Sadofsky, M. J., Hesse, J. E., & Gellert, M. (1995). Initiation of V(D)J recombination in a cell-free system. Cell, 81(6), 925-934.

van Kooten, C., & Banchereau, J. (2000). CD40-CD40 ligand. [Research Support, Non-U.S. Gov't

Review]. J Leukoc Biol, 67(1), 2-17.

van Kuppevelt, T. H., Dennissen, M. A., van Venrooij, W. J., Hoet, R. M., & Veerkamp, J. H. (1998). Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney. [Comparative Study

Research Support, Non-U.S. Gov't]. J Biol Chem, 273(21), 12960-12966.

van Lochem, E. G., van der Velden, V. H., Wind, H. K., te Marvelde, J. G., Westerdaal, N. A., & van Dongen, J. J. (2004). Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. Cytometry B Clin Cytom, 60(1), 1-13. doi: 10.1002/cyto.b.20008

Vankemmelbeke, M., Chua, J. X., & Durrant, L. G. (2016). Cancer cell associated glycans as targets for immunotherapy. Oncoimmunology, 5(1), e1061177. doi: 10.1080/2162402X.2015.1061177

Vasseur, J. A., Goetz, J. A., Alley, W. R., Jr., & Novotny, M. V. (2012). Smoking and lung cancer-induced changes in N-glycosylation of blood serum proteins. Glycobiology, 22(12), 1684-1708. doi: 10.1093/glycob/cws108

Veri, M. C., Gorlatov, S., Li, H., Burke, S., Johnson, S., Stavenhagen, J., . . . Koenig, S. (2007). Monoclonal antibodies capable of discriminating the human inhibitory Fcgamma-receptor IIB (CD32B) from the activating Fcgamma-receptor IIA (CD32A): biochemical, biological and functional characterization. Immunology, 121(3), 392-404. doi: 10.1111/j.1365-2567.2007.02588.x

Vestergaard, E. M., Hein, H. O., Meyer, H., Grunnet, N., Jorgensen, J., Wolf, H., & Orntoft, T. F. (1999). Reference values and biological variation for tumor marker CA 19-9 in serum for different Lewis and secretor genotypes and evaluation of secretor and Lewis genotyping in a Caucasian population. [Research Support, Non-U.S. Gov't]. Clin Chem, 45(1), 54-61.

Vidarsson, G., Dekkers, G., & Rispens, T. (2014). IgG subclasses and allotypes: from structure to effector functions. Front Immunol, 5, 520. doi: 10.3389/fimmu.2014.00520

Viret, F., Ychou, M., Lepille, D., Mineur, L., Navarro, F., Topart, D., . . . Chouaki, N. (2004). Gemcitabine in combination with cisplatin (GP) versus gemcitabine (G) alone in the treatment of locally advanced or metastatic pancreatic cancer: Final results of a multicenter randomized phase II study. Journal of Clinical Oncology, 22(14), 342s-342s.

Von Hoff, D. D., Ervin, T., Arena, F. P., Chiorean, E. G., Infante, J., Moore, M., . . . Renschler, M. F. (2013). Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med, 369(18), 1691-1703. doi: 10.1056/NEJMoa1304369

Wagner, A. D., Grothe, W., Haerting, J., Kleber, G., Grothey, A., & Fleig, W. E. (2006). Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. J Clin Oncol, 24(18), 2903-2909. doi: 10.1200/jco.2005.05.0245

Walport, M. J. (2001). Complement. First of two parts. N Engl J Med, 344(14), 1058-1066. doi: 10.1056/nejm200104053441406

Wang, P. H., Lee, W. L., Juang, C. M., Yang, Y. H., Lo, W. H., Lai, C. R., . . . Yuan, C. C. (2005). Altered mRNA expressions of sialyltransferases in ovarian cancers. Gynecol Oncol, 99(3), 631-639. doi: 10.1016/j.ygyno.2005.07.016

Wang, P. H., Lee, W. L., Lee, Y. R., Juang, C. M., Chen, Y. J., Chao, H. T., . . . Yuan, C. C. (2003). Enhanced expression of alpha 2,6-sialyltransferase ST6Gal I in cervical squamous cell carcinoma. Gynecol Oncol, 89(3), 395-401.

Wang, S. J., & Bourguignon, L. Y. (2006). Hyaluronan and the interaction between CD44 and epidermal growth factor receptor in oncogenic signaling and chemotherapy resistance in head and neck cancer. Arch Otolaryngol Head Neck Surg, 132(7), 771-778. doi: 10.1001/archotol.132.7.771

Wang, W., Singh, S., Zeng, D. L., King, K., & Nema, S. (2007). Antibody structure, instability, and formulation. J Pharm Sci, 96(1), 1-26. doi: 10.1002/jps.20727

Wang, W., Wang, E. Q., & Balthasar, J. P. (2008). Monoclonal antibody pharmacokinetics and pharmacodynamics. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't]. Clin Pharmacol Ther, 84(5), 548-558. doi: 10.1038/clpt.2008.170

Wang, Z., Wen, L., Ma, X., Chen, Z., Yu, Y., Zhu, J., . . . Li, Y. (2012). High expression of lactotriaosylceramide, a differentiation-associated glycosphingolipid, in the bone marrow of acute myeloid leukemia patients. Glycobiology, 22(7), 930-938. doi: 10.1093/glycob/cws061

Watanabe, M., Ohishi, T., Kuzuoka, M., Nudelman, E. D., Stroud, M. R., Kubota, T., . . . et al. (1991). In vitro and in vivo antitumor effects of murine monoclonal antibody NCC-ST-421 reacting with dimeric Le(a) (Le(a)/Le(a)) epitope. Cancer Res, 51(8), 2199-2204.

Watson, D. S., Endsley, A. N., & Huang, L. (2012). Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. [Research Support, N.I.H., Extramural

Review]. Vaccine, 30(13), 2256-2272. doi: 10.1016/j.vaccine.2012.01.070

Watson, N. F., Madjd, Z., Scrimegour, D., Spendlove, I., Ellis, I. O., Scholefield, J. H., & Durrant, L. G. (2005). Evidence that the p53 negative / Bcl-2 positive phenotype is an independent indicator of good prognosis in colorectal cancer: a tissue microarray study of 460 patients. World J Surg Oncol, 3, 47. doi: 10.1186/1477-7819-3-47

Watson, S. A., Morris, T. M., Crosbee, D. M., & Hardcastle, J. D. (1993). A hepatic invasive human colorectal xenograft model. [Research Support, Non-U.S. Gov't]. Eur J Cancer, 29A(12), 1740-1745.

Weerasinghe, P., & Buja, L. M. (2012). Oncosis: an important non-apoptotic mode of cell death. Exp Mol Pathol, 93(3), 302-308. doi: 10.1016/j.yexmp.2012.09.018

Weerasinghe, P., Hallock, S., Brown, R. E., Loose, D. S., & Buja, L. M. (2013). A model for cardiomyocyte cell death: insights into mechanisms of oncosis. Exp Mol Pathol, 94(1), 289-300. doi: 10.1016/j.yexmp.2012.04.022

Weichenthal, M., Siemann, U., Neuber, K., & Breitbart, E. W. (1999). Expression of complement regulator proteins in primary and metastatic malignant melanoma. J Cutan Pathol, 26(5), 217-221.

Weiner, L. M., Murray, J. C., & Shuptrine, C. W. (2012). Antibody-based immunotherapy of cancer. Cell, 148(6), 1081-1084. doi: 10.1016/j.cell.2012.02.034

Weller, S., Faili, A., Garcia, C., Braun, M. C., Le Deist, F. F., de Saint Basile, G. G., . . . Weill, J. C. (2001). CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. Proc Natl Acad Sci U S A, 98(3), 1166-1170. doi: 10.1073/pnas.98.3.1166

Westerink, M. A., Giardina, P. C., Apicella, M. A., & Kieber-Emmons, T. (1995). Peptide mimicry of the meningococcal group C capsular polysaccharide. [Comparative Study

Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. Proc Natl Acad Sci U S A, 92(9), 4021-4025.

Weston, B. W., Hiller, K. M., Mayben, J. P., Manousos, G. A., Bendt, K. M., Liu, R., & Cusack, J. C., Jr. (1999). Expression of human alpha(1,3)fucosyltransferase antisense sequences inhibits selectin-mediated adhesion and liver metastasis of colon carcinoma cells. Cancer Res, 59(9), 2127-2135.

Willems, A., Gauger, K., Henrichs, C., & Harbeck, N. (2005). Antibody therapy for breast cancer. Anticancer Res, 25(3A), 1483-1489.

Wolff, E. A., Schreiber, G. J., Cosand, W. L., & Raff, H. V. (1993). Monoclonal antibody homodimers: enhanced antitumor activity in nude mice. Cancer Res, 53(11), 2560-2565.

Wormald, M. R., Rudd, P. M., Harvey, D. J., Chang, S. C., Scragg, I. G., & Dwek, R. A. (1997). Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. Biochemistry, 36(6), 1370-1380. doi: 10.1021/bi9621472

Wortis, H. H., Teutsch, M., Higer, M., Zheng, J., & Parker, D. C. (1995). B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. Proc Natl Acad Sci U S A, 92(8), 3348-3352.

Wright, A., & Morrison, S. L. (1997). Effect of glycosylation on antibody function: implications for genetic engineering. Trends Biotechnol, 15(1), 26-32. doi: 10.1016/s0167-7799(96)10062-7

Wright, A. J., & Andrews, P. W. (2009). Surface marker antigens in the characterization of human embryonic stem cells. [Research Support, Non-U.S. Gov't

Review]. Stem Cell Res, 3(1), 3-11. doi: 10.1016/j.scr.2009.04.001

Wulf, G. G., Wang, R. Y., Kuehnle, I., Weidner, D., Marini, F., Brenner, M. K., . . . Goodell, M. A. (2001). A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid leukemia. Blood, 98(4), 1166-1173.

Xu, Z., Zan, H., Pone, E. J., Mai, T., & Casali, P. (2012). Immunoglobulin class-switch DNA recombination: induction, targeting and beyond. Nat Rev Immunol, 12(7), 517-531. doi: 10.1038/nri3216

Yamada, N., Chung, Y. S., Takatsuka, S., Arimoto, Y., Sawada, T., Dohi, T., & Sowa, M. (1997). Increased sialyl Lewis A expression and fucosyltransferase activity with acquisition of a high metastatic capacity in a colon cancer cell line. Br J Cancer, 76(5), 582-587.

Yamamoto, H., Saito, T., Kaneko, Y., Kersey, D., Yong, V. W., Bremer, E. G., . . . Moskal, J. R. (1997). alpha2,3-sialyltransferase mRNA and alpha2,3-linked glycoprotein sialylation are increased in malignant gliomas. Brain Res, 755(1), 175-179.

Yamane-Ohnuki, N., Kinoshita, S., Inoue-Urakubo, M., Kusunoki, M., Iida, S., Nakano, R., . . . Satoh, M. (2004). Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. Biotechnol Bioeng, 87(5), 614-622. doi: 10.1002/bit.20151

Yamashita, T., Wada, R., Sasaki, T., Deng, C., Bierfreund, U., Sandhoff, K., & Proia, R. L. (1999). A vital role for glycosphingolipid synthesis during development and differentiation. Proc Natl Acad Sci U S A, 96(16), 9142-9147.

Yang, M. C., Hsu, F. L., Handa, K., Hakomori, S., Lee, M. H., Liu, L. Y., . . . Chang, T. H. (2009). Human monoclonal antibody GNX-8 directed to extended type 1 chain: Specific binding to human colorectal cancer. Int J Cancer. doi: 10.1002/ijc.25131

Yang, Y. M., & Chang, J. W. (2008). Current status and issues in cancer stem cell study. Cancer Invest, 26(7), 741-755. doi: 10.1080/07357900801901856

Yates, A. J. (1986). Gangliosides in the nervous system during development and regeneration. Neurochem Pathol, 5(3), 309-329.

Ye, F., Li, Y., Hu, Y., Zhou, C., & Chen, H. (2010). Stage-specific embryonic antigen 4 expression in epithelial ovarian carcinoma. [Comparative Study

Research Support, Non-U.S. Gov't]. Int J Gynecol Cancer, 20(6), 958-964. doi: 10.1111/IGC.0b013e3181e6fee1

Ye, F., Li, Y., Hu, Y., Zhou, C., Hu, Y., & Chen, H. (2010). Stage-specific embryonic antigen 4 expression in epithelial ovarian carcinoma. Int J Gynecol Cancer, 20(6), 958-964. doi: 10.1111/IGC.0b013e3181e6fee1

Yogeeswaran, G., & Salk, P. L. (1981). Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. Science, 212(4502), 1514-1516.

Yong, P. F., Salzer, U., & Grimbacher, B. (2009). The role of costimulation in antibody deficiencies: ICOS and common variable immunodeficiency. Immunol Rev, 229(1), 101-113. doi: 10.1111/j.1600-065X.2009.00764.x

Yoo, E. M., Wims, L. A., Chan, L. A., & Morrison, S. L. (2003). Human IgG2 can form covalent dimers. J Immunol, 170(6), 3134-3138.

Yoshida, T., Mei, H., Dorner, T., Hiepe, F., Radbruch, A., Fillatreau, S., & Hoyer, B. F. (2010). Memory B and memory plasma cells. Immunol Rev, 237(1), 117-139. doi: 10.1111/j.1600-065X.2010.00938.x

Young, D. (2007). A. R. Inc.

Yuasa, N., Zhang, W., Goto, T., Sakaue, H., Matsumoto-Takasaki, A., Kimura, M., . . . Fujita-Yamaguchi, Y. (2010). Production of anti-carbohydrate antibodies by phage display technologies: potential impairment of cell growth as a result of endogenous expression. [Research Support, Non-U.S. Gov't]. J Biol Chem, 285(40), 30587-30597. doi: 10.1074/jbc.M110.107284

Yun, Y. H., Eteshola, E., Bhattacharya, A., Dong, Z., Shim, J. S., Conforti, L., . . . Watts, N. (2009). Tiny medicine: nanomaterial-based biosensors. Sensors (Basel), 9(11), 9275-9299. doi: 10.3390/s91109275

Yuriev, E., Farrugia, W., Scott, A. M., & Ramsland, P. A. (2005). Three-dimensional structures of carbohydrate determinants of Lewis system antigens: implications for effective antibody targeting of cancer. Immunol Cell Biol, 83(6), 709-717. doi: 10.1111/j.1440-1711.2005.01374.x

Zarnegar, B., He, J. Q., Oganesyan, G., Hoffmann, A., Baltimore, D., & Cheng, G. (2004). Unique CD40-mediated biological program in B cell activation requires both type 1 and type 2 NF-kappaB activation pathways. [Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, Non-P.H.S.

Research Support, U.S. Gov't, P.H.S.]. Proc Natl Acad Sci U S A, 101(21), 8108-8113. doi: 10.1073/pnas.0402629101

Zhang, D., Zhang, G., Hayden, M. S., Greenblatt, M. B., Bussey, C., Flavell, R. A., & Ghosh, S. (2004). A toll-like receptor that prevents infection by uropathogenic bacteria. Science, 303(5663), 1522-1526. doi: 10.1126/science.1094351

Zhang, S., Cordon-Cardo, C., Zhang, H. S., Reuter, V. E., Adluri, S., Hamilton, W. B., . . . Livingston, P. O. (1997). Selection of tumor antigens as targets for immune attack using immunohistochemistry: I. Focus on gangliosides. Int J Cancer, 73(1), 42-49.

Zhang, S., Zhang, H. S., Reuter, V. E., Slovin, S. F., Scher, H. I., & Livingston, P. O. (1998). Expression of potential target antigens for immunotherapy on primary and metastatic prostate cancers. Clin Cancer Res, 4(2), 295-302.

Zhang, X. (2013). Regulatory functions of innate-like B cells. Cell Mol Immunol, 10(2), 113-121. doi: 10.1038/cmi.2012.63

Zhao, J., Weng, X., Bagchi, S., & Wang, C. R. (2014). Polyclonal type II natural killer T cells require PLZF and SAP for their development and contribute to CpG-mediated antitumor response. Proc Natl Acad Sci U S A, 111(7), 2674-2679. doi: 10.1073/pnas.1323845111

Zhou, X., Hu, W., & Qin, X. (2008). The role of complement in the mechanism of action of rituximab for B-cell lymphoma: implications for therapy. Oncologist, 13(9), 954-966. doi: 10.1634/theoncologist.2008-0089

Zuckier, L. S., Chang, C. J., Scharff, M. D., & Morrison, S. L. (1998). Chimeric human-mouse IgG antibodies with shuffled constant region exons demonstrate that multiple domains contribute to in vivo half-life. Cancer Res, 58(17), 3905-3908.

Appendix

1. *(Chua et al., 2015)*

   Chua, J. X., M. Vankemmelbeke, et al. (2015). "Monoclonal Antibodies Targeting LecLex-Related Glycans with Potent Antitumor Activity." Clin Cancer Res 21(13): 2963-2974.

2. *(Vankemmelbeke, Chua, & Durrant, 2016)*

   Vankemmelbeke, M., J. X. Chua, et al. (2016). "Cancer cell associated glycans as targets for immunotherapy." Oncoimmunology 5(1): e1061177.