The University of Nottingham

Development of tools to target Antigen through Mannose Receptor

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Thesis submitted to the University of Nottingham for the

Degree of Doctor of Philosophy

School of Molecular Medical Sciences

July 2011

Abstract

Dendritic cells (DC) are unique antigen presenting cells which play a major role in antigen presentation and initiation of the immune response by regulating B- and T- cell activation. Antigen targeting to DC receptors is an effective, safe and specific method for vaccine development. The mannose receptor (MR) is an endocytic receptor expressed by subpopulations of DC and antigen targeting through MR leads to enhanced antigen uptake and presentation to T-cells. This makes MR a favourite receptor for the development of vaccines against diseases that require T-cell immunity such as cancer and viral infections. This project sought to develop tools to target antigens through MR and investigate their ability to induce T-cells activation *in vitro* and *in vivo*.

We have used three approaches to deliver antigen through MR; (i) MR-specific mAbs: 5D3 and 6C3, have been chemically linked to the melanoma epitope TRP-2, (ii) MR-specific chimeric antibodies carrying several model antigens have been generated by using genetic engineering and (iii) Glycopolymers and the suitable antigens such as a shorter version of model antigen ovalbumin (OVA), with and without N-glycosylation sites have been generated and characterised. Glycopolymer-OVA conjugates were prepared by chemical coupling but it requires further optimization. The binding efficiency of anti-MR antibodies has been assessed using ELISA and BIACORE and the glycopolymers have been tested for their interaction with MR. Immunisations were performed with anti-MR mAb-TRP2 conjugates which induced TRP-2 specific $CD8^+$ T-cells activation and improved humoral response. Due to limitations in this approach in terms of chemical coupling being an inefficient method and the potential involvement of Fc recetors (FcRs), chimeric Abs fused to model antigens and bearing mutated Fc were generated. These chimeric Abs, have been tested for their ability to induce T-cell activation *in vitro* and

in vivo. But the progress has been hampered due to the labile nature of these reagents.

In future, anti-MR chimeric Abs will be used to generate anti-MR single chain antibodies carrying OVA (ScFv-OVA) and the glycopolymer project will be taken up Dr. Manovani Giuseppe (School of Pharmacy, University of Nottingham). It will involve further optimization of chemical coupling of glycopolymers to a-glycosylated OVA-mini protein, and the *in vitro* Ag presentation assay to investigate whether glycopolymers mediated Ag targeting of APC enhance T-cells activation. These further studies would greatly benefit the understanding of the mechanisms associated with the elicitation of immune resposes as a result of Ag targeting through MR. Anti-MR reagents generated in this study along with appropriate adjuvant could be exploited to target malarial, cancerous and viral Ags for robust T-cell activation against these infectious diseases. On the other hand, the role of MR in homeostasis and allergy has been already established, and the anti-MR reagents generated in this study can be used to target allergens and self-Ags to APCs in an attempt to induce tolerance.

Acknowledgments

I would like to take this opportunity to thank Dr Luisa Matrinez-Pomares, School of Molecular and Medical sciences, under whose supervision this work has been carried out. Her continued support, guidance and encouragement throughout this project have been much appreciated.
I would also like to thank my co supervisor professor Lindy Dorrant for her guidance and help throughout the project specifically in MR-mediated targeting of TRP-2 project. I am also thankful to Dr. Ian Spendlove and Dr. Victoria Pudney (City Hospital, department of clinical oncology) for their support in the same project. Professor Weng Chan is also thanked for providing support in chemical coupling of IgG molecules with TRP-2.

I have been fortunate to work with various brilliant people in various groups namely: Lectin biology, Allergy group and Virology group. I would also like to thank my friends and colleagues Umut Gazi and Sonali Singh who have supported me during these last four years. Dr Alex and Mr Shafiq-ur-Rehman are being acknowledged for their technical advice on DNA cloning and DNA sequence analysis. I am grateful to Dr. Richard Please and Dr. Richard Macintosh for their help in "restriction cloning and generation of chimeric antibody".

Dr Giuseppe Mantovani, School of pharmacy, for providing me the opportunity to work with glycopolymers.

I would like to thank higher education commission of Pakistan for providing financial assistance for this project.

Last but not least I wish to thank my mother and father and the rest of family and friends who provided me with moral support, guidance and inspiration to work harder and achieve big goals in my life.

Abbreviations

Ab	Antibody
Ag	Antigen
AP	Alkaline phosphatase
APC	Antigen Presenting Cell
ATRP	Atom transfer radical polymerization
BCG	Bacillus Calmette-Guérin
BM	Bone-Marrow
BMDCs	Bone-Marrow derived Dendritic Cells
BSA	Bovine Serum Ablumin
CCR7	CC-Chemokine Receptor 7
CDRs	Complementarity Determining Regions
CHO-cells	Chinese Hamster Ovary cells
CR	Cysteine-Rich
CRD	Carbohydrate Recognition Domain
CTLD	C-Type Lectin Like Domain
DC	Dendritic Cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNGR-1	Dendritic Cell NK Lectin Group Receptor-1
EDTA	Ethylenediaminetetra-acetic acid
EER	Early Endoplasmic reticulum
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FACS	Flourescence Activated Cell Sorting
FBS	Fetal Bovine Serum
Fc	Fc portion of immunoglobulin
FCA	Freund's Complete Adjuvant
FcR	Fc Receptor
FCS	Fetal Calf Serum

FDA	Food And Drug Administration
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GSP	Gene Specific Primers
HBsAg	Hepatitis B Surface Antigen
HEK293T	Human Embryonic Kidney 293 Cells
HEL	Hen Egg Lysozyme
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
ICAM3	Intercellular Adhesion Molecule-3
IEF	Isoelectric Focusing
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
Kda	Kilo Dalton
LN	Lymph Node
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MDAs	Melanocyte Differentiation Antigens
MHC	Major Histocompatibility Complex
MPLA	Monophosphoryl Lipid A
MR	Mannose Receptor
OD	Optical Density
OMLs	Oligomannose-Coated Liposomes
OVA	Ovalbumin
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PE	Phycoerytherin
PEI	Polyethylenimine
PRR	Pattern Recognition Receptors

Q-TOF	Quadrupole Time-Of-Flight
RACE	Rapid Amplification Of cDNA Ends
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylamde Gel Electrophoresis
SEM	Standard Error of the Mean
SPR	Surface Plasmon Resonance
TAE buffer	Tris-Acetate-EDTA
TAP	Transfer Associated With Antigenic Processing
TCEP	Tris(2-Carboxyethyl)Phosphine
TLR	Toll-Like Receptors
TRP-2	Tyrosinase-Related Protein 2
UPM	Universal Primers Mix
v/v	Volume/Volume
w/v	Weight/Volume
WT	Wild Type

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1 General Introduction

1.1 Vaccines

Diseases caused by infections are a major cause of death in humans and vaccination has been an important medical intervention to treat infections for last 200 years (Pashine et al., 2005). Vaccines are biological preparations used to establish or improve immunity to a particular disease. The term 'vaccine' derived from Edwar Jenner's use of cow pox: Latin *variolæ vaccinæ,* adapted from the Latin *vaccinus*, from *vacca* cow (Peter J. Delves, 2011).

The earliest vaccines were developed in China based on the concept of variolation in which a person is infected with the weak virus obtained from variola scabs as a form of inoculation to immunise against small pox (Plotkin, 2004). Later, Jenner observed that deliberate infection with cow pox virus; which is nonvirulent in humans, induced mild disease and consequent elicitation of immunity against smallpox (Plotkin, 2004). Though variolation was banned for sometime due to some fatal reactions of small pox, this idea of deliberate infection to protect against infectious disease led to the eradication of smallpox in the twentieth century (Pardoll, 2002).

Jenner's works eventually lead to Louis Pasteur's attenuated vaccines. Pasteur came upon this idea when he was working on chicken cholera caused by *Pasteurilla multicoda* (Plotkin, 2005). He infected chickens with an old culture which did not cause disease. These pre-infected chickens showed resistance when infected with a new culture of *P. multicoda*. On the basis of this observation Pasteur hypothesised that pathogens could be attenuated by

environmental stress such as high temperature and chemicals, which was confirmed by his work on rabies and anthrax (Plotkin, 2005).

1.2 Immunotherapy

Immunotherapy is wider term defined as the treatment of diseases by manipulating the immune system. It is of two types: active and passive immunotherapy. Self-limiting infectious diseases are easily controlled by traditional active vaccination strategies. Treatment of chronic infectious diseases or cancer is currently the main objective of immunotherapy, and it requires better understanding of the immune systems in terms of its regulatory mechanisms, identification of appropriate antigen and optimization of the interaction between antigen presenting cells (APC) and T-cells (Waldmann, 2003) (see section 1.5.6).

Passive immunotherapy refers to the transfer of pre-made immunological molecules, mostly antibodies after to naïve patients. The concept of passive immunity was introduced by Koch's laboratory, where they injected animals with a small dose of diphtheria toxin. The serum containing anti-toxin antibodies was collected from the immunised animals to be administered to diphtheria patients providing passive immunity (Waldmann, 2003).

The discovery of monoclonal antibodies (Kohler and Milstein, 1975) revolutionised passive immunotherapy; Muronomab-CD3 was the first monoclonal antibody approved by FDA to prevent organ allograft rejection (Group, 1985). In order to reduce immunogenicity monoclonal antibodies have been modified into chimeric and humanized antibodies and are being used to

cure various different diseases. Some mAbs such as Gemtuzumab ozogamicin (Kreitman, 1999) and Ibritumomab tiuxetan (Witzig et al., 2002) linked with toxin and radionuclide have been used to treat leukaemia and lymphoma respectively.

Active immunotherapy involves long lasting changes in the immune system that lead to the induction of acquired immune response against the vaccine and establishment of memory. Active immunotherapy is the most desirable and widely used form of immunotherapy which, as mentioned above, started from Edward Jenner's discovery of the protective effect of deliberate infection with cow pox virus (Plotkin, 2005). This strategy of active immunotherapy led to the development of vaccines against many infectious diseases as described in Table.1.1.

Time scale	*Development in vaccination*
1796	Jenner introduces vaccinia (cowpox) immunisation to prevent subsequent small pox infection.
1879-1886	Louis Pasteur introduces first laboratory-weakened infectious agent (chicken cholera bacterium) and shortly there after develops weakened rabies for active immunisation.
1888	Emile Roux and Alexander Yersin isolate toxin from Diphthea.
1890	Emil Von Behring and Shibasabo Kitasato in Koch's laboratory find that injecting diphtheria toxin into animal produces a serum containing an antitoxin that provides passive anti-diphtheria immunity to people.
1900	Paul Ehrlich suggests that molecules that react with tumours could play a key role in cancer therapy, presaging antibody –mediated passive immunotherapy.
1954-1955	Jonas Salk and Albert Sabin introduce killed and live attenuated polio vaccines that soon lead to the elimination of poliomyelitis.
1965	IgG anti-D (anti-RH) is administered to prevent of RH immunisation and thus prevents erythryblastosis fetalis; this is a transiation of the basic insight that passive administration of a specific IgG antibody inhibits the active production of that antibody.
1975	George Kohler and Cesar Milstein develop hybridoma technology for monoclonal antibody generation.
1977	Smallpox is declared eradicated through vaccination.
1982	The first report of a successful use of a monoclonal antibody to treat a human neoplasm (patient specific anti-idiotype antibody to treat B-cell

	lymphoma) is reported.
1986	The first monoclonal antibody, muromonab –CD3 (orthoclone OKT3), is approved by the FDA.
1986	The first humanized antibody is produced by replacing the complementarity regions in a human antibody with those of a mouse.
1986-2000	IL-2, IFN-α, IFN-β and IFN-γ are proved for usse in the treatment of neoplasia, hepatitis and multiple sclerosis.
1988-1991	The methodology for isolating tumour antigens recognized by CTLs is introduced; the first human antigen from melanoma patients identified by CTLs is isolated.
1997	The first humanized monoclonal antibody (daclizumab, zenapax) is approved by the FDA.
1997	The first monoclonal antibody (rituximab, rituxan) for the treatment of malignancy is approved.
1998	An antibody to TNF-α (infliximab, remicade), and p75 TNF receptor linked to the Fc of IgG1 (etanercept, Enbrel) are approved for use in the treatment of rheumatoid arthritis and Crohn disease.
2000	The first toxin-linked monoclonal Ab (Gemtuzumab ozogamicin Mylotarg) is approved by the FDA.
2002	The first radinuclide-linked monoclonal Ab (ibritumomab tiuxetan, Zevalin) is approved by the FDA.
2007	**Tositumomab** is a IgG2a anti-CD20 monoclonal antibody derived from immortalized mouse cells used for the treatment of follicular lymphoma (Jacene et al., 2007).
2008	**Cetuximab** is a chimeric mAb, an epidermal growth factor receptor (EGFR) inhibitor, for treatment of metastatic colorectal cancer and head and neck cancer (Yonesaka et al., 2011).
2009	**Palivizumab** is a humanized monoclonal antibody directed against an epitope in the A antigenic site of the F protein of respiratory syncytial virus (RSV), used in the prevention of RSV infections in infants (Wang et al., 2008).
2009	**Alemtuzumab** is a monoclonal antibody used in the treatment of chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL) and T-cell lymphoma (Schnitzler et al., 2009).
2010	**Catumaxoma** is a bispecific rat-mouse hybrid monoclonal antibody which is used to treat malignant ascites, a condition occurring in patients with metastasizing cancer (Linke et al., 2010).

Table 1.1: Key developments in vaccination along with the time scale. (Waldmann, 2003).

Classic vaccines which are based on the principal of active immunisation contain either the live, attenuated or a portion of disease causing agent (bacteria, virus or toxin) along with an adjuvant. Vaccines are normally administered with a priming dose in order to initate the immune response

followed by the two to three booster doses which induces not only the effector cells but also memory immune cells. The general classification of vaccines is shown in figure 1.1.

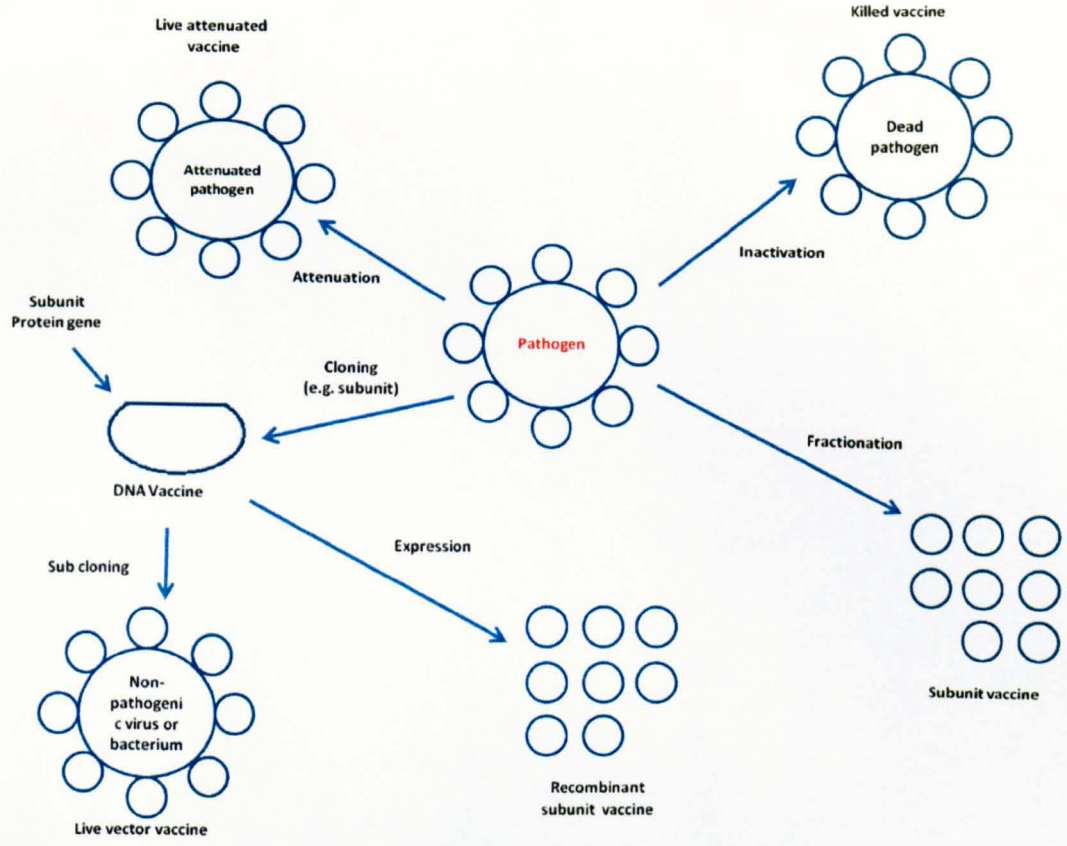


Figure 1.1: Classical approaches to develope killed, attenuated, inactivated and recombinant subunit vaccines from pathogens. (Peter J. Delves, 2011).

1.2.1.1 Killed vaccines

These vaccines are composed of pathogens killed by either chemicals or heat and induce effective humoral immune response as they include all the epitopes and virulence factor of the pathogen, however during deactvation it should be ensured that important antigenic parts of the organism are not destroyed. As they are composed of dead organisms they do not cause the side reactions

associated with live vaccines such as replication and reacquisition of virulence within the host. As compared to bacteria and viruses parasitic worms and protozoa are extremely difficult to grow in order to produce killed vaccines. Examples of killed vaccines are influenza, cholera and inactivated poliomyelitis (Salk) vaccines (Peter J. Delves, 2011). Moreover these vaccines have longer shelf life and are easier to handle and store. Killed vaccines also cause side reactions such as in the case of pertussis vaccine which causes inflammation at the site of injection and leads to fever due to the presences of endotoxins in the vaccine preparation (Sidey et al., 1989).

1.2.1.2 Live / attenuated vaccines

Natural infection or the infection with the modified live organism leads to the induction of most effective and long lasting cellular and humoral immunity without using adjuvants. Live vaccines are administered through the natural route of infection i.e. where the natural infection occurs, and needs fewer no of re-inoculations (Roberts, 2004). Live vaccines are attenuated or their virulence capacity is reduced either by repeated passages (10-1000 times) *in vitro* or by genetic manipulation as in the case of the sabin oral polio and flu mist vaccines (Roberts, 2004). On the other hand a non-pathogenic partner of the organism which shares immunoprotective epitopes can be used to induce immunity against natural pathogens. For example Bacillus Calmette-Guérin (BCG) vaccine in which attenuated *Mycobacterium bovis* is used to protect against natural infection caused by *M. tuberculosis* (Peter J. Delves, 2011).

Although live vaccines are very effective to induce immunity, they can also cause severe side reactions such as inflammation at the site of inoculation and as they replicate inside the host can revert back to a virulent strain by genetic recombination. Moreover, live vaccines are difficult to store as their half life is short which makes it difficult to keep organisms alive for long periods of time (Wilson-Welder et al., 2009).

1.2.1.3 Subunit vaccines

Subunit vaccines are composed of only a portion of pathogen and could be either an immunogenic peptide or DNA encoding the peptide. As compared to other types of vaccines it is safer and does not lead to side reactions associated with live or killed vaccines as it does not include virulence factors such as endotoxins and other TLR agonists (see section 1.4) which induce innate immune responses. On the other hand subunit vaccines are not very immunogenic and need multiple doses of the vaccines along with the appropriate adjuvant to trigger the immunity. Even with the adjuvant subunit vaccines leads to the induction of humoral responses but fail to induce cell mediated immunity (Wilson-Welder et al., 2009). Subunit vaccines can be improved to induce cell mediated immunity by using different antigen targeting approaches in combination with different adjuvants, to induce cell mediated immunity (Singh and O'Hagan, 2003).

Recombinant viruses such as modified vaccinia Ankara strain can be used as a vector to deliver antigens associated with pathogens and to induce immunity. These recombinant viral vectors include constructs which are replication

deficient, non-integrating, stable and easy to prepare. Examples include Hepatitis B surface antigen (HBsAg) and influenza hemagglutinin when expressed using recombinant vaccinia virus result in the induction of immunity and protection against hepatitis B virus and influenza infection in chimpanzee and mice, respectively as shown in figure 1.2. (Peter J. Delves, 2011).

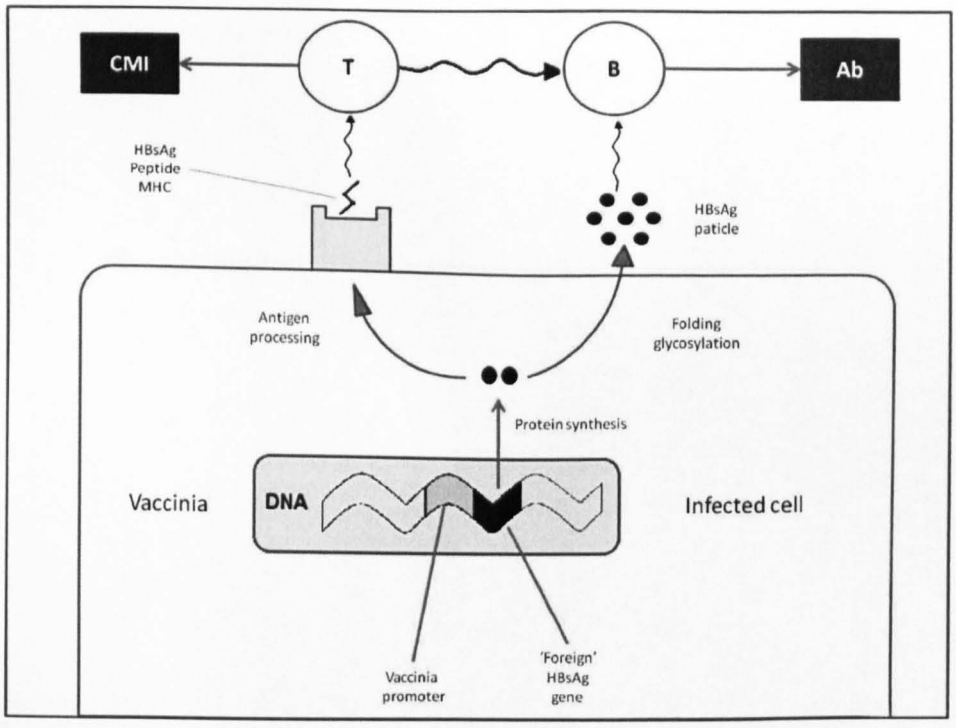


Figure 1.2: Hepatitis B surface antigen (HBsAg) vaccine using vaccinia virus as a vector to deliver antigen (Peter J. Delves, 2011).

Antibody-mediated targeting of Ag or subunit vaccine, to APC is another approach to develop T-cell immunity. Different receptors expressed by APC such as DEC205 (CD205) dendritic cell-Specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), DC NK lectin group receptor-1 (DNGR1) and mannose recptor (MR) have been tested in order to deliver antigen to APC and to elicit antigen-speficic immunity (see section 1.6.2).

1.3 Routes of vaccine administration

As described in section 1.2 different vaccines are associated with different adverse reactions at the site of injection. In order to reduce the side reaction and maximize immunogenicity of the vaccines, selection of an appropriate route of vaccine administration is needed. Normally vaccines are administered through subcutaneous, intramuscular and mucosal routes (Cook, 2008, Denis et al., 2007). Conventionally, all vaccines other than BCG and typhoid were given subcutaneously until vaccination led to an extra inflammation at the site of injection(Volk et al., 1954).

I.F Cook showed that for all vaccine categories such as live attenuated, inactivated whole cell, toxoid and subunit vaccines, intramuscular administration leads to better immune outcome and reduced side reaction as compared to subcutaneous injection (Cook, 2008). Increased reaction at the site of reaction associated with subcutaneous route of vaccine can be explained by two theories: (i) according to first theory numerous sensory neurons are present in subcutaneous as compared to muscles (Lindblad, 2004) and (ii) inflammatory cells present in the skin might be involved in the inflammation at the site of injection (Laurichesse et al., 2007).

Administration of vaccines through the mucosa is performed either through nose or mouth and induces protective immune response in both mucosal level and systemically. Vaccines against the microbes causing cholera, diarrhea and typhoid or pathogens which infect in the gastric tract are given through the oral or rectal route and vaccines against respiratory pathogens may be be

administered though the nose. Mucosal vaccines which are currently being used are the sabin oral polio vaccine (OPV) (Dowdle et al., 2003), rota virus vaccine and cold-adapted influenza vaccine (Belshe et al., 2000). Currently with a few exceptions most of the vaccines are administered either through the subcutaneous or intramuscular routes as Ag dose and its deposition can be controlled at the site of injection. Ag deposition continuosly supply Ag to APCs which is difficult to achieve by the intradermal route. Other than the route of vaccine administration other factors such as the type of Ag, type of adjuvant and time of vaccination are also important to achieve an appropriate immune response.

1.4 Role of adjuvants in the development of vaccines

The word adjuvant is derived from Latin verb *adjuvare* which means to help or aid and it may be defined as compoment of vaccine formulation which stimulates prolonged specific immune response to a vaccine without having any antigenic effect (Lindblad et al., 1997).

Only antigen which is transported into secondary lymphoid organ in sufficient amount and for sufficient time leads to the induction of effective immunity. On the other hand if the Ag persists in the secondary lymphoid organ or transported there in excess for long periods of time it leads to antigen specific T-cell anergy (Zinkernagel, 2000). Subcutaneous injection of bovine serum albumin, hen egg lysozyme and fowl gamma globulin does not lead to the induction of T-cell or antibody response due to rapid degradation of the foreign antigenic protein as their half life is very short (Weigle, 1961). Adjuvants

entrap the antigen in poorly metabolized or slowly degradable materials and protect antigen from degradation. Morover, adjuvants provides an Ag depot which facilitate its transport to secondary lymphoid organs for long time and in sufficient quantity as to induce an effective immune response. Aluminium phosphate and aluminium hydroxide known as alum are commonly used in human vaccines (Wilson-Welder et al., 2009). Freund's complete adjuvant (FCA) is one of the most effective adjuvants. It is composed of water in oil emulsion containing killed mycobacteria and it is highly reactogenic. It is used as an adjuvants in animal vaccines but not in humans as it induces granuloma formation at the site of injection. Freund's incomplete adjuvant contains only water oil emulsion and is recommended to be employed in influenza and HIV vaccine (Vogel, 2000). The adjuvants which are currently used along with the licensed vaccines are given in Table 1.2.

Humans, US	***Humans, United Kingdom and European Union***	***Live stock worldwide (general categories)***
Aluminum hydroxide, Aluminum phosphate, Potassium aluminum sulfate(alum)	Aluminum hydroxide, Aluminum phosphate, Potassium aluminum sulfate(alum) Calcium phosphate MF-59 (squalene in Fluad) ASO4 (liposome formulation containing MPLA and QS-21) (FENDrix, servaix)	Aluminum hydroxide, Aluminum phosphate, Potassium aluminum sulfate(alum) Saponin QS-21 Oil emulsions paraffins, Mineral oil, lanolin, Squalene, ISA-70, Montanide (IMS) Glycerin

Table 1.2: Vaccine adjuvants currently used along with the licensed vaccines (Adapted from Wilson-Welder et al., 2009).

Other than depot effect, adjuvants also interact with pattern-recognition receptors (PRR) expressed on APC and provide danger signal as as it occours

during an active infection. These adjuvants could be derived by pathogens that express conserved structures such as pathogen associated molecular patterns (PAMPs) (Wilson-Welder et al., 2009, O'Hagan and Valiante, 2003). PRR recognizies PAMPs and induce innate immune response by activating the complement pathways and intracellular signalling leading to inflammatory reponses required to clear pathogens (Janeway and Medzhitov, 2002, Pulendran and Ahmed, 2006). PRR are found in three different compartments such as body fluids, cell membrane and cytoplasm. PRR found in body fluids are involved in the the activation of complement pathway (Gasque, 2004). PRR associated with cell membrane include toll like receptor (TLR), scavenger receptors, MR and other C-type lectins which help microbial uptake by phagocytosis and activation of intracellular signalling pathways (Lee and Kim, 2007).

TLRs are best to generate intracellular signalling and shape the acquired immunity. In mammals all TLRs are type-I transmembrane glycoproteins containing an extracllular domain for ligand recognition and a cytoplasmic Toll/IL-1R homology (TIR) domains to initiate intracellular signalling (Akira and Takeda, 2004). Four different TIR domain containing adaptor molecules such as MyD88, TIRAP, TRIF and TRAM are recruited in different combinations to initiate signalling pathways (Akira and Takeda, 2004) as shown in figure 1.3. Toll-like receptors (TLR) recognise pathogen-associated molecular patterns (PAMPS) that act as warning signs of infection. Consequently TLR are considered pattern recognition receptors (PRR) (Pasare and Medzhitov, 2004). Engagement of the TLR with the PAMPS results in the

DC maturation due to the increased cytokines production on its surface which is very important for the development of the immunity (Hemmi and Akira, 2005). Cross presentation (Burgdorf et al., 2008) and the antigen presentation through MHC-II pathway by the DCs increased in the presence of endotoxins (Blander and Medzhitov, 2006). So, PRR on the DCs play an important role in the Ag presentation are critical for the development of the immunity.

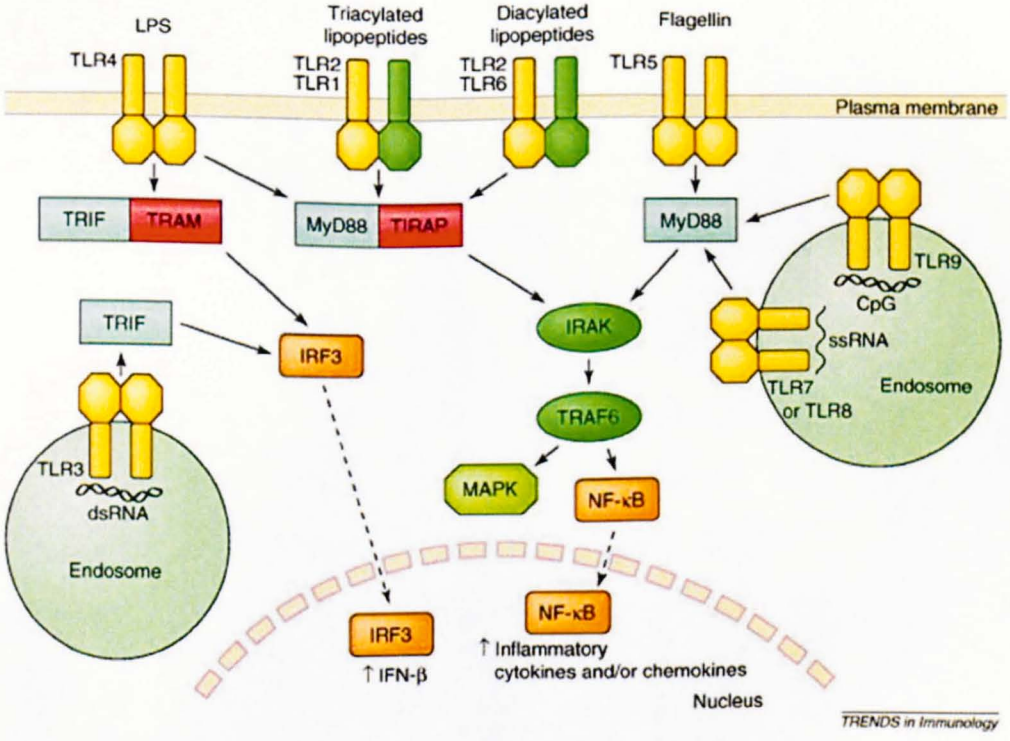


Figure 1.3: Overview of major TLR signaling pathways.

All TLRs signal through MyD88, with the exception of TLR3. TLR4 and the TLR2 subfamily (TLR1, TLR2, TLR6) also engage TIRAP. TLR3 signals through TRIF. TRIF is also used in conjunction with TRAM in the TLR4–MyD88-independent pathway. Dashed arrows indicate translocation into the nucleus. LPS, lipopolysaccharide; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; IRF3, interferon regulatory factor-3. **Adapted from Duin et al. (van Duin et al., 2006).**

LPS are the component of gram negative bacterial cell wall and signals through endotoxin receptor TLR-4 through MyD88 and TRIF mediated pathways and

results in the maturation and migration of DCs to secondary lymphoid organ. The unique feature of the LPS as an adjuvant is that it can be administered through any route and different time than vaccine but still it keeps its adjuvanticity. LPS is used as an adjuvant in animals only and it is not permitted in humans due to its toxicity which is due its signaling through MyD88 pathway which result in the induction of IFN-γ, IL-1β, IL-6 and other molecules associated with toxicity of LPS (Mata-Haro et al., 2007). Due to the toxic effects of the adjuvants only limited adjuvants are permitted to be used with human vaccines. LPS can be detoxified or its toxicity can be reduced from 100 to 1000 time by removing phosphate group, sugar moiety and ester linked fatty acid group (Johansson et al., 2004). MPLA is such a molecule derived from Salmonella which has almost 0.1% inflamatory cytotoxicity as compared to LPS (Evans et al., 2003). MPLA signals through endotoxin receptor TLR-4 by TRIF pathway and as an adjuvant it was found equally potent as LPS to induce CD4+ and CD8+ T-cells proliferation. Morevoer, MPLA retained the induction of type I interferon by TLR4/TRIF mediated signaling which is important to generate an antiviral state in mosT-cells by T-cell clonal proliferation (Kolumam et al., 2005).

Prokaryotic DNA includes unmethylated CpG dinuleotides in high frequency which are recognized by the innate immune system of the vertebrates (Krieg, 2002). These sequences signals through TLR-9 (located on endosomal memebrane) by MyD88 pathway (Takeshita et al., 2001). CpG motifs activate DCs by upregulating the expression of CD80, CD86, MHC-II, and transient secretion of type I interferon (Verthelyi and Zeuner, 2003, Kerkmann et al.,

2003). CpG adjuvant is found very effective to induce immunity against very weak Ags such as melanoma, malarial Ags (Diwan et al., 2002).

1.5 Dendritic cells (DC) and vaccine development

1.5.1 Dendritic cells (DCs)

Dendritic cells are professional APC. They play a major role in the initiation and control of immune responses by regulating T and B lymphocytes activation. These cells are strategically positioned throughout the body in an immature state, surveying the tissues for invading pathogens and are unique in antigen capturing, processing, and presentation as compared to other antigen-presenting cells (Banchereau and Steinman, 1998).

1.5.2 Location of DCs

DCs are derived from bone marrow progenitors and circulate in the blood as immature precursors prior to migration into peripheral tissues. Within different tissues, DCs differentiate and become active in the taking up and processing of Ags. The location of the DCs inside the body is unique to capture the foreign antigens such as body surfaces like the skin, pharynx, upper oesophagus, vagina, ectocervix and anus, and at mucosal surfaces, such as the respiratory and gastrointestinal systems (Niess et al., 2005).

In steady state conditions, in most tissues DCs are immature; unable to stimulate the T-cells due to the lack of required accessory signals such as CD40, CD54, and CD86, but they are highly equipped with the antigen capturing Fcγ and Fcε recptors to uptake the antigens (Banchereau and Steinman, 1998). Upon antigen uptake and appropriate stimulation, DCs

undergo further maturation and migrate to secondary lymphoid tissues where they present Ag to T-cells and induce an immune response (Satthaporn and Eremin, 2001).

1.5.3 Migration of DCs to lymphoid tissues

DCs are highly mobile, they need to migrate from the periphery to the T-cells area of the secondary lymphoid organs through lymphatic vessels in order to present Ag to Tcells (Randolph et al., 2005). Body movements drive the lymph inside the lymphatic vessels in a unidirectional way from the periphery to the lymph nodes (LNs) and ultimately to the heart (Swartz, 2001). Mature DCs expressing the chemokine receptor, CC-cemokine receptor 7 (CCR7) move towards the lymphatic vessels through chemotaxis as shown in figure 1.4 (Ohl et al., 2004, Sallusto et al., 1998). Little is known about the expression of CCR7 but it is correlated with the upregulation of major hisocompatibility molecule II (MHC-II) and co-stimulatory molecules which occurs during DCs maturation (Sallusto et al., 1998). It is proposed that the lymphatic endings secrete the CCL19 and CCL21 chemokines and CCR7 positive DCs move towards these molecules in a concentration gradient dependent manner and taken up by the terminal lymphatic vessels. Once inside the LN, DCs present antigen to the T-cells and die locally. DCs do not leave the efferent lymph which is opposite to the movement of the T-cells (Kamath et al., 2002).

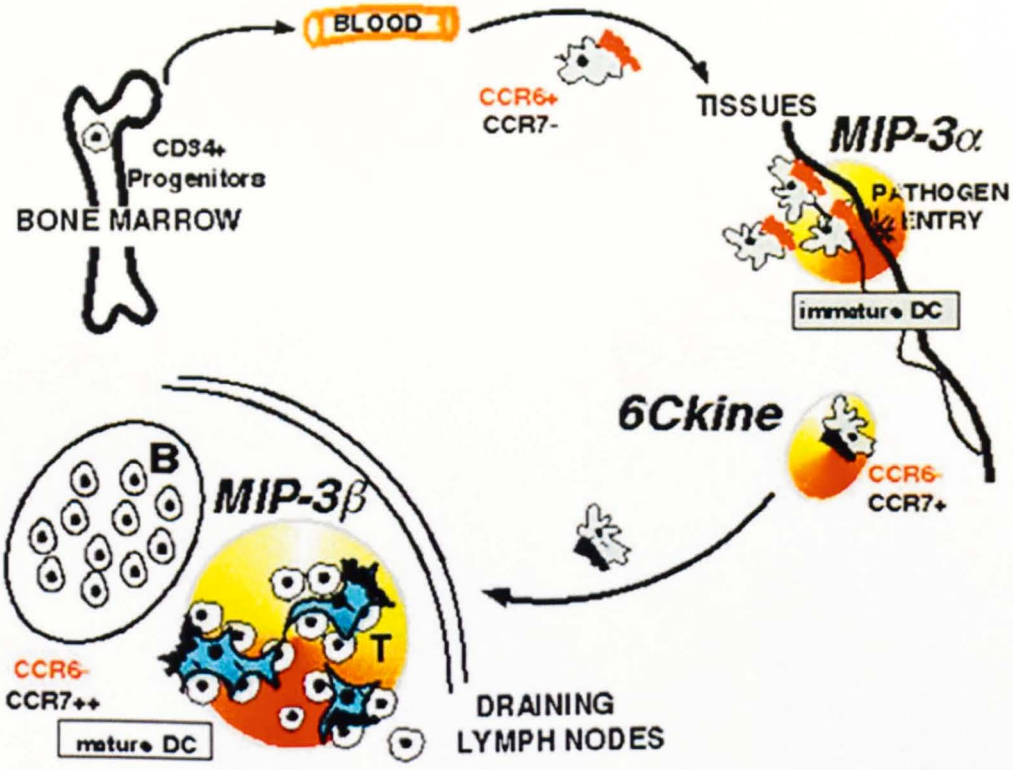


Figure 1.4: The critical role of chemokines in the migration of dendritic cells to lymphoid tissue.

Precursor and immature dermal DC or langerhans cells that display CCR6 are attracted by the epithelium that expresses the specific ligand MIP-3a. Upon antigen capture and activation, dermal DC detach from keratinocytes by downregulating E-cadherin. Meanwhile CCR6 is replaced by CCR7, whose ligands are (a) 6Ckine, which is expressed on lymphatic vessel walls, and (b) MIP-3b, which is expressed in the T-cell areas of lymphoid organs. This may guide the maturing DCs to the T-cell areas where they will start to produce chemokines that attract lymphocytes. **Adapted from Banchereau et al. (Banchereau et al., 2000).**

1.5.4 Antigen uptake and processing

DCs uptake exogenous Ag by different mechanisms such as pinocytosis or receptor mediated-endocytosis which result in the targeting of the soluble Ag to distinct intracellular compartment and antigen presentation to $CD4^+$ or $CD8^+$ T-cells (Burgdorf et al., 2007). Materials taken up by the APC by pinocytosis is targeted to the endocytic pathway (Burgdorf et al., 2007) which is composed of

three increasingly acidic compartments: early endosome, late endosome and finally lysosome. The acidic nature of the endocytic cycle as well as the hydrolytic enzymes in the lysosomal compartment degrades the endocytosed material into shorter oligopeptides appropriate for recognition by class II MHC molecules.

Classical studies showed that endogenous proteins are processed through the cytosolic pathway and the peptides derived from the endogenous antigens are transported to the endoplasmic reticulum where these are loaded on the MHC class I molecule and transferred to the cell surface for antigen presentation. On the other hand recent studies showed that APC especially the DCs have the ability to process exogenous antigen and present on the MHC I molecules. This mechanism is known as cross presentation and is important to induce the immune response against the viruses and tumours which are not infecting the APC (Heath and Carbone, 2001).

1.5.5 Cross-presentation

The molecular mechanism of the cross presentation is not that clear as the MHC I restricted presentation of endogenous antigens. In the cytoplasm, endogenous antigens are digested into the shorter antigenic peptides by the proteosome which are taken to the endoplasmic reticulum by transfer associated with antigenic processing (TAP), where they are loaded on the MHC I molecules and transferred to the cell surface through Golgi apparatus via the secretory pathway for presentation to cytotoxic T lymphocytes (Burgdorf et al., 2008).

When analysed *in vitro*, DCs and macrophages were found as the key cells capable of cross presentation by exposing different bone marrow derived APCs to exogenous antigens. However, the cross priming ability of DC *invivo* has been deomonstrated by two methods: Firstly DCs isolated from animals were exposed to the Ags exvivo and then injected into the mice which led to the activation of $CD8^+$ T-cell immunity due to cross presentation (Pozzi et al., 2005). Secondly, deletion of APCs from transgenic mice (CD11c-DTR) using diphtheria toxin, result into the inhibition of cross presentation which indicates that DCs present exogenous Ag on MHC-I molecules and are one of the key cells involved in cross presentation (Jung et al., 2002).

DCs in mice are heterogenous and their subtypes differ in morphology and functionality. DCs located in the secondary lymphoid tissues can be both resident and migratory DCs (Ardavin, 2003). In spleen resident DCs are divided in two subsets: CD8+ and C8- DCs based on the expression of CD8αα homodimers. There are many differences between these cell subsets with CD8+ DCs preferentially cross presenting the exogenous Ag on MHC-I molecules while on the other hand CD8- DCs do it on the MHC-II molecules. Differential Ag processing in these cells was confirmed by microarray studies which showed that $CD8^+$ DCs express more proteins involved in MHC-I presentation and CD8- DCs express more proteins which are involved in the MHC-II presentation (Dudziak et al., 2007). Compared to resident DCs very little is known about the cross priming abilitites of the migratory DCs. Amongst different skin-derived DCs CD103+ DCs are the main migratory

subtype which captures the viral and self antigen from the skin and cross present (Bedoui et al., 2009).

The mechanism involved in the in the cross-presentaion of exogenous Ag have been of great interest due to its implications in vaccine development against cancer and viral infections. Different groups have tried to elucidate it but still the intracellular compartments involved in this process are not well defined. So, different theories of cross presentation have been suggested on the basis of intracellular pathways used as shown in figure 1.5. Different pathways of cross-presentation are described below.

1.5.5.1 Vacuolar pathway

This pathway of cross-presentation is distinct from other pathways as it is independent to TAP and cytosolic proteasome. So in this pathway the peptides for the cross presentation are generated in the phagosome by cysteine protease cathepsin S, the MHC-I molecule is transported to the phagosome through either a tyrosine-based signal or provided by endoplasmic reticulum (ER) through its fusion with phagosome (Shen et al., 2004). Fusion of ER with phagosome is controversial as Touret et al., failed to demonstrate fusion of ER with phagosome by different methods which show that fusion of ER with phagosome is an artifact in this pathway of cross-presenataion. So, either MHC-I molecule reaches peptide by signal or get ER MHC-I molecule by some method other than fusion (Touret et al., 2005).

1.5.5.2 Phagosome to cytosol pathway

In the phagosome to cytosol pathway of cross presentation, antigen from the phagosome is transported into the cytosol for digestion by the cytosolic proteasome and peptides are transported back into the phagosome to be loaded on MHC-I molecule (Kovacsovics-Bankowski and Rock, 1995). Phagosome fuse with ER which provides several proteins including MHC-I molecule, tapasin, chaperones and Sce61. Sec61 plays a role for the escape of Ag from the phagosome into the cytosol for digestion. The peptides are then carried with the help of TAP into ER or back into the phagosome as phagosome after fusion with ER includes all essential proteins for loading of peptide on MHC-I molecules (Guermonprez et al., 2003).

1.5.5.3 Endosome to ER and ER associated degradation pathway.

In the endosome to ER and ER-associated degradation pathway of cross presentation soluble antigens are internalized by DCs and transported into the ER. Ag escapes into the cytosol using the ER-associated degradation pathway. Ag is digested into peptides by cytosolic proteasome which are taken up by TAP and re-tarnsported into ER where they are loaded on MHC-I molecules and cross presented (Imai et al., 2005).

1.5.5.4 Mannose Receptor (MR) to Early Endoplasmic Reticulum (EER) pathway

Burgdorf, Scholz et al. (2008) proposed a cross presentation system on the basis of their experimental findings in which they have suggested that the soluble model Ag such as Ovalbumin (OVA) is taken up by the DCs with the

help of mannose receptor and transported to the stable early endosome compartment. From the early endosome OVA is exported to the cytoplasm where it is degraded into OVA peptides by the proteasome. Peptides are then reimported into the same early endosome compartment with help of TAP, loaded on the MHC I molecule and transported directly (Not through the GA and secretory pathway) to the cell membrane for presentation to CTL.

On the otherhand, Nadine et al. (2009) found that a continuous supply of MHC-I peptide is required in order to prime cytotoxic T lymphocytes *in vivo*. In order to provide a continous supply, Ag is stored in a lysome-like organelle that functions as a depot (van Montfoort et al., 2009) but it is clearly distinct from EER compartment as described above (Burgdorf et al., 2008).

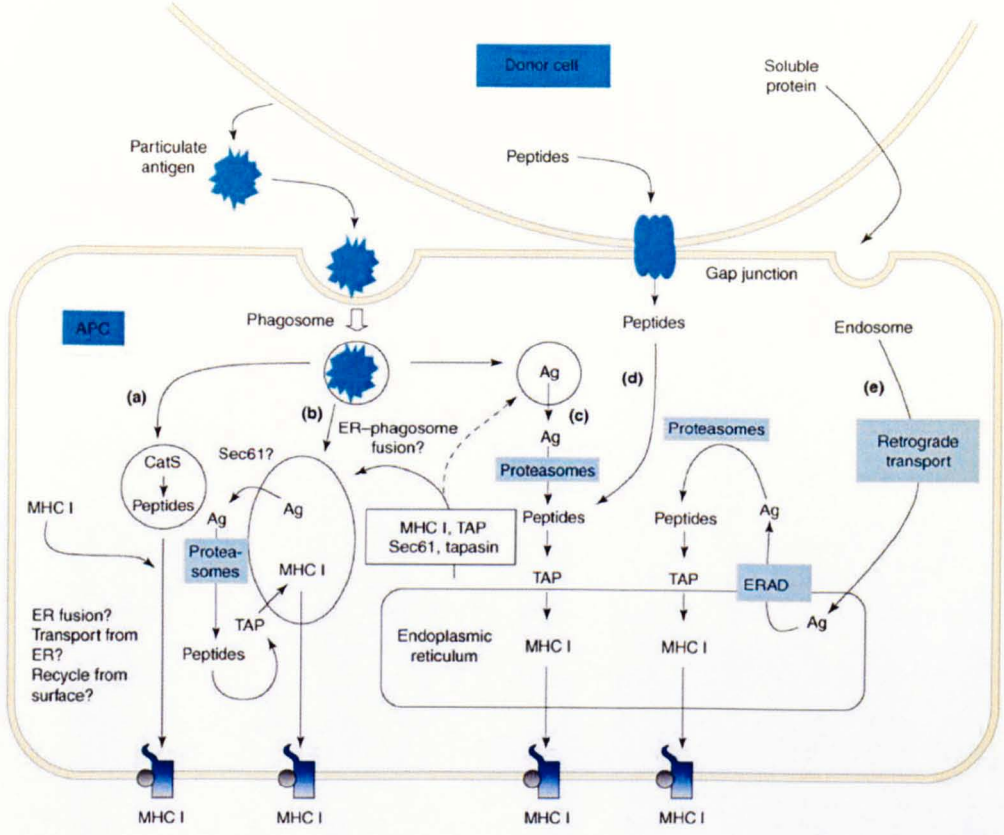


Figure 1.5: Different mechanisms of cross presentation.

(a) The vacuolar pathway. MHC class I molecules sample peptides that are generated in phagosomes in a TAP-independent manner. **(b)** Phagosome-to-cytosol-to-phagosome pathway. Phagosomes acquire MHC class I, TAP, Sec61 and other ER molecules through fusion with the ER. Internalized antigen is exported (possibly by Sec61) to the cytosol, hydrolyzed by proteasomes and the resulting peptides are then re-imported into phagosomes by TAP, where they bind MHC class I molecules. **(c)** Phagosome-to-cytosol pathway. Antigen internalized into phagosomes is exported to the cytosol, hydrolyzed by proteasomes and the resulting peptides are then transported to MHC class I molecules in the ER by TAP. **(d)** GAP junction pathway. APCs acquire peptides from other cells through GAP junctions. **(e)** Endosome-to-ER pathway. Antigen in endosomes is transported into the ER and then degraded in the cytosol by the ER associated degradation pathway (ERAD). The resulting peptides are transported to class I molecules in the ER by TAP. **Adapted from Shen et al. (Shen and Rock, 2006)**

1.5.6 DC maturation

DC maturation refers to the upregulation of costimulatory molecules, enhancement in their antigen presenting function, and the expression of chemokine receptors such as CCR7 which direct the movement of DCs to the secondary lymphoid organs (Tacken et al., 2007). In the absence of maturation

stimulus when DCs encounter the Ag they induce tolerance when they capture self and environmental Ag (Hawiger et al., 2001). However in the presence of maturation stimulus such LPS a massive migration of the DCs is observed from the skin, heart, kidneys and intestines (Roake et al., 1995).

Toll-like receptors (TLRs) are the best characterized pathogen recognition receptors which recognise the pathogen associated molecular patterns (PAMPS) that act as warning signs of infection.(Akira et al., 2006) and (Kawai and Akira, 2005). For DCs, TLR agonists have adjuvant effect on the antigen presentation by inducing the increase in antigen uptake, MHC I expression and loading, and also by stimulating the expression of co-stimulatory molecules. From vaccination point of view, targeting the immature DC in the absence of maturation stimulus is good strategy to treat allergy, chronic inflammatory and autoimmune diseases but on the other side to treat cancer and other infectious diseases it is essential to induce the immune response by targeting the mature DCs. Time and the route of the administration of the maturation stimuli are of the crucial importance to induce the maturation of DCs (Tacken et al., 2007), which modulate the Ag processing by lowering the pH of endocytic, activating proteolysis, and transporting peptide MHC complexes to the cell surface for the presentation (Trombetta and Mellman, 2005). Maturation stimuli should be applied systemically as the stimuli applied too long before or after the Ag administration results in the decrease of cross presentation (Wilson et al., 2006). Some DC receptors such as CD205 completely loose antigen uptake capacity upon full maturation, therefore, it is better to target the antigen and maturation stimuli simultaneously (Butler et al., 2007). To activate DC it is

better to use the combination of maturation stimuli as targeting of human DCs through mannose receptor in the presence of R848 (signals through TLR7 and TLR8) and poly I:C (signals through TLR3) results in inducing the T helper cells and CTL responses (Ramakrishna et al., 2007).

Mature DCs (mDCs) carrying the Ag move towards the secondary lymphoid tissues such as local lymph nodes (LN) and present Ag and activate T-cells in the presence of endotoxoin, in the T-cell area of the LN. Activated T-helper cells are involved in the production of the antibodies in order to remove the Ag. On the other hand active cytotoxic T-lymphocytes and leave the LN throught the efferent lymph and kill the infected cells (Steinman, 2001). But the Ag presented by the DCs in the absence of the infection signal such as endotoxins results in the T-cell deletion and leads to the peripheral tolerance (Janeway and Bottomly, 1994).

1.6 Exploitation of dendritic cells for vaccine development

1.6.1 Ex-vivo loading of dendritic cells and their use as adjuvants

Inaba et. al., first described the role of DCs as adjuvants. In this study, DCs isolated from mouse spleen were primed with the specific antigen overnight. DCs processed and presented the antigen epitopes onto MHC molecules and Ag-loaded DCs were then injected into mice, which led to Ag specific T-cell sensitization and development of immunity. The immune response was robust when the mouse was challenged again with the DC pulsed with the same antigen, due to the presence of memory cells (Inaba et al., 1990).

Methods of preparing DCs have changed since they were considered trace cell types of the immune system, when *in vitro* protocols were employed to grow DCs from their progenitors. Inaba et al., identified and reported clusters of DCs from cultures of mouse blood supplemented with GM-CSF (Inaba et al., 1992b). Bone-marrow being the precursor of DCs, they were soon thereafter identified in the blood culture, and a method was thus described to grow large numbers of DCs from bone-marrow (BM) cultures of mice supplemented with GM-CSF (Inaba et al., 1992a). These new methods of DC culture paved the way to further characterise DCs and investigate their clinical application. In order to investigate the capacity of BM-derived DC (BMDC) to be used as an adjuvant to induce immunity against infectious diseases, BMDC were pulsed with Bacillus Calmette-Guerin organism, and induced a strong T-cell response when injected *in-vivo* (Inaba et al., 1993).

To investigate the role of DCs as adjuvant in humans, they are prepared from the culture of blood monocytes supplemented GM-CSF and IL-4 (Romani et al., 1994). Later, a method to generate mature DCs from human blood was described in which they used macrophage conditioned media containing essential maturation factors (Romani et al., 1996). DCs generated with this method were clinically more potent as an adjuvant. When these cells were fed with B-cell lymphoma and tumour lysate, and injected as a vaccine into patients suffering from B-cell lymphoma (Hsu et al., 1996) and melanoma (Nestle et al., 1998) respectively, induced Ag specific imunity.

DCs have been used directly as an adjuvant in immunotherapy (Mohamadzadeh and Luftig, 2004) but with limited success as the

methodology is very expensive, complex and time consuming. Additionally, antigen-loaded DCs accumulate at the site of injection and only 1-2% of the antigen pulsed DCs reach the local lymphoid organ. Due to these limitations alternative approaches have been developed to target DCs directly *in vivo* in the presence or absence of infectious signals (Shortman et al., 2009, Tacken et al., 2006).

1.6.2 Development of vaccine by antibody mediated targeting of antigens.

Antigen targeting with the help of antibodies (Ab) against receptors found on DCs is the most specific and reliable method to develop vaccines. In this method, antibodies specific for the receptors expressed on DC are used as a vehicle to deliver Ag. Given the rarity of DCs in the body, it is better to use antibodies to deliver specific antigen as it results into greater uptake and presentation of the antigen by the DCs (Keler et al., 2007). For instance, the efficiency of OVA uptake and presentation was increased 1000-fold for MHC-I molecule and 300 fold for MHC-II molecule when targeted using anti-DEC205 antibody, as compared to non-specific targeting of OVA (Bonifaz et al., 2002).

DCs express endocytic receptors and Ab-mediated Ag delivery through them leads to the accumulation of Ag into specialised compartments of the cell where they are loaded on to the relevant MHC molecules to elicit the Ag specific immune response. Common receptors used successfully for vaccine development through this methodology are complement receptors, integrins, members of the immunoglobulin superfamily and C-type lectin receptors (CLRs) (Keler et al., 2007). The most common CLRs used in targeting studies

are the MR, CD205 and DC-specific intercellular adhesion molecule-3 (ICAM3)-grabbing non integrins (DC-SIGN). Ag-targeting through different receptors results in different immune outcomes due to different intracellular receptor routing, signalling pathways and expression patterns (Tacken et al., 2007). Here, we will mainly focus on the potential of MR as a candidate for vaccine development by its specific targeting using anti-MR mAb, anti-MR chimeric antibody and glycopolymers.

1.6.2.1 DEC205

DEC-205 belongs to MR family, and it was first identified as a receptor on mouse DC. It is a 200 KDa glycoprotein composed of a cysteine-rich domain, fibronectin type II domain, and 10 CTLD domains for which natural ligands have not yet been identified. It is an endocytic receptor which takes up the antigen cargo and delivers it to the intracellular compartment of DCs, where it is loaded on MHC molecule and presented to T-cells (East and Isacke, 2002).

Ab-mediated antigen delivery through DEC-205 has been found to be 100-fold more efficient as compared to non-specific targeting. Model antigens such as HEL (Hawiger et al., 2001) and OVA (Bonifaz et al., 2002) have been fused with anti-DEC205 antibody; and when immunised in the presence of an appropriate adjuvant like anti-CD40 agonist, elicited strong $CD4^+$ helper T-cell antibody response (Boscardin et al., 2006) and strong $CD8^+$ T-cell immunity (Bonifaz et al., 2004). Different antigens of common infectious diseases such as the circumsporozite protein of malaria parasite (Boscardin et al., 2006), gig protein of HIV (Bozzacco et al., 2007) and nuclear antigen of Epstein-Barr virus (Gurer et al., 2008) have been targeted through DEC-205 in the presence

of anjuvant and result into strong antigen specific immunity. On the other hand, in the absence of infectious signal antigen targeting through DEC-205 lead to antigen specific T-cell tolerance. In this respect, ß cell antigen targeting result in deletion of autoreactive $CD8^+$ T-cells, which causes type 1 diabetes mellitus (Mukhopadhaya et al., 2008).

1.6.2.2 DNGR-1

C-type lectin 9a (Celc9a) is a C-type lectin like molecule expressed on the surface of $CD8^+$ mouse cDCs and BDCA-3 human DC subtype. It is a type II transmembrane receptor having one extracellular C-type lectin like domain (CTLD). DC NK lectin group receptor-1 (DNGR-1) is characterised as a member of C-type lectin of the NK receptor group and it was identified on mouse $CD8\alpha^+$ cDCs and partially on pDCs. It is an endocytic receptor of molecular weight 51 KDa found in dimmers. $CD8\alpha^+$ DCs are specialised in cross presentation and the induction of CTL based immunity and DNGR-1 selectively expressed on these DCs (Sancho et al., 2008). Ag-targeting through this receptor can therefore be used to treat some chronic infectious diseases such malaria, HIV and cancer. The potential of DNGR-1 for Ag-targeting and the vaccine development portal was assessed by immunisation of mice with anti-DNGR-1, coupled with melanoma antigen S1 along with anti-CD40 adjuvant. The mice were challenged with B16 melanoma cells and anti-DNGR-1 targeting resulted in strong and long lasting T-cell memory, which leads to eradication of B16 melanoma lung pseudometastases (Sancho et al., 2008).

1.6.2.3 DC-SIGN

DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) and its equilent DC-SIGNR belongs to the family of type II transmemranne CLRs initially discovered during search of DC surface receptors involved in DC and T-cell interaction. The identified receptor showed Ca dependent binding with ICAM-3 and composed of a single extracellular C-type lectin like domain, a transmembrane and cytoplasmic tail. CLRs such as Dectin-1, Dectin-2, Langerin and MGL which signals either directly or indirectly through their interaction with some TLRs are expressed on APCs and largely used for the antigen targeting and have a lot of scope to treat different infectious diseases (Tacken et al., 2006).

1.6.2.4 MR

Mannose receptor (MR, CD205) was the first discovered member of the MR-family, found to be involved in the clearance of endogenous glycoproteins. MR was thus named because of its lectin activity to proteins glycosylated with mannose, fucose or N-acetyl glucosamine. MR demonstrates structural homology with other receptors, most significantly DEC205 (CD205), Endo180 and the phospholipase A2 receptor. MR is an endocytic receptor and type-I protein of molecular weight 175 KDa. It is composed of three extracellular domains: a Cysteine-rich (CR) domain that shows calcium independent binding with sulphated sugars; a Fibronectin type II (FNII) domain that is the most conserved region of MR and binds with collagen; and eight C-type lectin-like carbohydrate recognition domains that bind in a calcium-dependent manner with sugars or proteins terminated in mannose, fucose and N-acetylaglucosamine. Moreover, it contains a transmembrane domain and a

short C-terminal cytoplasmic tail that mediates receptor internalisation (East and Isacke, 2002) as shown in figure 1.6.

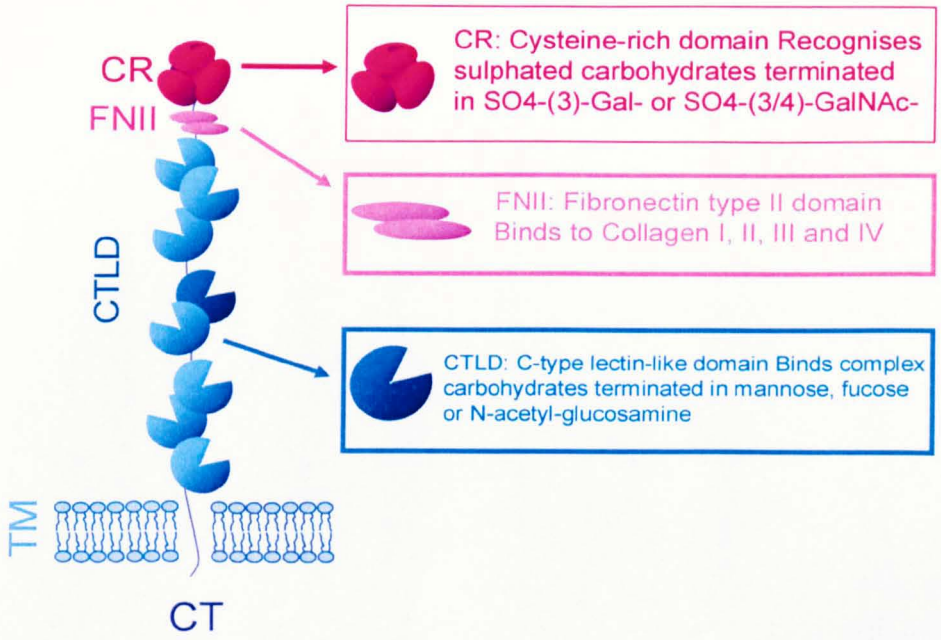


Figure 1.6: Domain structure of MR and their ligands.

MR consists of three extracellular domains, CR: cysteine-rich domain, FNII: domain-containing fibronectin type II repeats, CTLD: C-type carbohydrate-like domains and TM: transmembrane domain, CT: cytoplasmic tail.

1.7 Antigen targeting through MR

Targeting of model antigen OVA through MR results in the cross presentation and activation of T-cell responses against OVA is the fascinating finding which make MR a strong candidate for the development of therapeutic vaccines against infections which need T-cell immunity such as viral and tumour infection (Burgdorf et al., 2008). MR is an efficient endocytic receptor expressed by DC (CD11c+, MHC-II+, CD11b+, DEC205+, CD8α$^-$) in T-cell areas of peripheral LN in the presence of selected TLR agonists in mouse (McKenzie et al., 2007) and, possibly, humans (Martinez-Pomares et al. unpublished). Importantly, it appears that the fate of Ag internalised through

the MR is dictated by the type of MR ligand and Ag presenting cell used, and, probably, by the differential engagement of additional PRR in each experimental model.

1.7.1 Antibody mediated MR targeting

Rather than using the mannosylated or mannan linked Ags, antigen delivery by using anti-MR Ab is the most specific and efficient method to deliver Ag to APCs (Keler et al., 2007). Ramakrishna et al. first described MR targeting by using human anti-MR mAB (B11) fused with melanoma Ag, pmel17 (B11-pmel17). In this study monocyte derived DCs were fed with B11-pmel17 and co-cultrured with T-cells in order to generate Ag specific $CD4^+$ and $CD8^+$ effector T-cells. The reactivity of the effector T-cells was assessed by chromium release assay and they were found cytotoxic towards gp100+ HLA matched melanoma targets. This study demonstrated that the antibody-mediated targeting of exogenous tumour Ag through MR leads to the Ag presentation in context of MHC-I and MHC-II molecules and result in the induction of cytotoxic and helper T-cells (Ramakrishna et al., 2004). In order to perform in-vivo studies transgenic mice expressing hMR were generated and immunized with B11-OVA fusion proteins. B11-OVA targeted OVA through MR and efficiently presented to $CD4^+$ ad $CD8^+$ Tcells which led to the induction of Ag specific humoral and cellular immunity. In order to assess T-cell effector response mice were challenged with melanoma cells transfected OVA and hMR transgenic mice immunized with B11-OVA along with CpG induced OVA specific tumour immunity while WT mice failed to induce protective immunity (He et al., 2007). Recently, Tsuji et al. fused a cancer testis Ag NY-ESO-1 with anti-MR hmAb B11 (B11-NY-ESO-1) and found

that this construct efficiently target MR on APCs and result in antigen specific induction of $CD4^+$ and $CD8^+$ T-cells derived form PBMCs of cancer patients. These findings make MR-ESO fusion proteins a strong candidate to assess its potential for clinical cancer vaccine trials in cancer patients expressing NY-ESO-1 Ag (Tsuji et al., 2010).

1.7.2 Chemical glycosylation of antigens to facilitate MR-mediated Ag targeting

Mannosylation of Ags is a promising strategy to target DCs efficiently through MR and development of Ag specific immunity. Although antibody mediated targeting of Ags is the most specific and efficient method to target DCs and the development of vaccines but the generation of the antibodies is an expesive, time taking and immunogenic in many patients. Synthetic carbohydrates can be used alternatively to target DCs and the development of vaccines (Adams et al., 2008). Tan et al. chemically linked 2-6 mannose residues with the Ag petides and showed that mannosylated peptides or proteins were 200 to 10,000 times more efficient in inducing MHC-II restricted T-cell proliferation (Tan et al., 1997). In another study 6 different types of carbohydrates were conjugated with model Ag OVA by using cross-linker molecule SMCC. Carbohydarte-OVA conjugates increased the efficiency of antigen presentation by DCs via MHC-I and MHC-II molecules and leads to a 50 fold enhancement of Ag presentation to $CD4^+$ T-cells and 10 fold to $CD8^+$ T-cells (Adams et al., 2008).

1.7.3 MR-mediated targeting of glycosylated proteins expressed in fungi

Fungi have a natural ability to glycosylate proteins with mannose residue. Lam et al. generated recombinant glycosylated OVA Ags containing N an O-linked glycosylation in the yeast *Pichia pastoris* which were found more potent to induce OVA specific $CD4^+$ T-cell proliferation as compared to unglycosylated OVA proteins expresseid in *Escherchia coli (Lam et al., 2005).* Most of the licensed vaccines for humans are based on the induction of antibody mediated immune response. However the induction of cell mediated arm of the immune response is essential to provide protection against tumours and viral infections. In order to induce CTL immunity Stubbs et al. used recombinant *Saccharomyces cerevisiae* yeast expressing tumour or HIV-1 Ag. *In vivo* immunisation of recombinant yeast expressing OVA provided protection against tumour formation in E.G7-OVA tumour model in which mouse EL-4 lmyphoma cells transfected with OVA have beed employed (Stubbs et al., 2001).

Luong et al. assessed the effect of fungal N and O-lniked glycosylation on antigen specific $CD8^+$ T-cell proliferation (Luong et al., 2007). During glycosylation in *P pastoris*, the N-linked glycan structure has high mannose configuration as compared to O-linked glycans consist of only 2-3 mannose residues (Trimble et al., 2004). Fungal O-linked glycosylation of OVA was found to increase T-cell proliferation while N-linked glycosylation led to decreased T-cell proliferation which may be due to the antigen uptake by different mannose receptors (MRs). Mannosylated proteins target MRs such as

MMR (CD206) and DC-SIGN expressed on DCs. Oligosacharides containing terminal mannose binds MMR and deliver Ag into EER where it is cross presented the Ag on MHC-I molecule (Burgdorf et al., 2007) while the Ags associated with high mannose structures binds with DC-SIGN and delivered to late endosome and MHC-II presentation pathway (Guo et al., 2004).

1.7.4 MR-mediated delivery of Ag by using liposomes with associated sugars

Liposomes are good carriers for the vaccines and drug delivery also less they are immunogenic and non-toxic which makes them an attractive vaccination vector (Altin and Parish, 2006). Liposomes structures are flexible and could be manipulated in terms of its composition and the type of cargo (Ag, adjuvant or cytokines) to generate CTL immunity. Enhanced phagocytosis and stability of liposomes in the blood circulation are required to induce liposome mediated CTL immunity. In order to stabilize the structures of liposomes and to increased the retention time of liposomes in the blood, PEG-modified liposomes have been employed which greatly enhanced the generation of $CD8^+$ T-cell responses (Lasic et al., 1991). To increase phagocytosis liposomes have been coated with carbohydrates which facilitated the delivery liposomes vesicles to APCs through MRs which led to more phagocytosis and effective induction of CTL immunity (Sallusto et al., 1995). Kojima et al. evaluated the potential of oligomannose-coated liposomes (OMLs) to deliver tumour Ag to APCs and generate effective anti-tumour immunity. Mice immunized with OMLs carrying OVA Ag showed strong cytotoxic activity against E.G7-OVA melanoma cells and reduced the tumour growth. Similar tumour regression was observed when EL4 tumour cell lysate was also used as antigen in OML based

immunisation. This study indicates that OMLs-based vaccinations lead to the induction of antigen specific CTL immunity and can be clinically usefull in cancer immunotherapy (Kojima et al., 2008).

1.7.5 Glycopolymers to deliver antigen through MR

Gylcopolymers and their application in the development of vaccine by antigen targeting to APCs through MR is described in chapter 6.

1.8 Aim of the project

The basic aim of this project is to dissect how the interaction between MR and a MR ligand-Ag complex influences Ag processing and T-cell differentiation. To address this question we propose the following aims:

1. Generation of recombinant proteins consisting of the model Ag ovalbumin (OVA) fused to the binding site of the anti-MR mAb 5D3 and determination of the nature and magnitude of $CD4^+$ T-cell and $CD8^+$ T-cell responses elicited by 5D3:OVA *in vitro* and *in vivo* in the presence or absence of selected ligands for Toll-like receptors.
2. Chemical coupling of anti-MR mAbs and their isotype control to the melanoma Ag TRP-2 and assess their potential to induce TRP-2 specific CD 8 T-cell response in vitro and in vivo when immunised in the presence of adjuvant.
3. Conjugation of recombinant OVA mini-protein containing important $CD4^+$ and $CD8^+$ epitopes to different novel glycopolymers to analyse their ability to induce T-cell activation *in vitro.*

Our results will be greatly helpful in the development of antigen delivery methods suitable for robust T-cell activation.

2 Materials and methods

2.1 General Materials

2.1.1 Mice

Wild type (WT) C57BL/6, MR-/- mice on a C57BL/6 background (Lee et al., 2002) and BALB/C mice were bred, at BMSU under specific pathogen-free conditions while wild type (WT) mice were purchased from Charles River. In all experiments, mice between 7 to 10 weeks of age were used according to the local animal handling and experimentation guidelines of The University of Nottingham.

2.1.2 Media

1- R10 medium

RPMI 1640 (Gibco Invitrogen, UK)

10% volume/volume (V/V) fetal calf serum (FCS) (Gibco Invitrogen, UK)

100U/ml Pencillin-100μg/ml Streptomycin (Sigma-Aldrich, UK)

2 mM L-glutamine (Gibco Invitrogen, UK)

For dendritic cells culture R10 media was supplemented with mouse GM-CSF (R&D sytems UK) and also FBS used in this medium was low endotoxin, filtered instead of heat inactivated high quality FBS purchased from Sigma (Catalogue no. F6178).

2- DMEM10 medium

DMEM Glutamax plus medium (Gibco Invitrogen, UK)

10% volume/volume (V/V) fetal calf serum (FCS) (Gibco Invitrogen, UK)

100U/ml Pencillin-100μg/ml Streptomycin (Sigma-Aldrich, UK)

2mM L-glutamine (Gibco Invitrogen, UK)

3- OPTI-MEM medium

Opti-mem Glutamax plus (Gibco Invitrogen, UK)

100U/ml Pencillin-100µg/ml Streptomycin (Sigma-Aldrich, UK)

4- F12 medium

F12 medium Glutamax plus (Gibco Invitrogen, UK)

100U/ml Pencillin-100µg/ml Streptomycin (Sigma-Aldrich, UK)

10% volume/volume (V/V) fetal calf serum (FCS) (Gibco Invitrogen, UK)

5- IMDM complete mdium

Iscove's Modified Dulbecco's Media (IMDM) medium (Gibco Invitrogen, UK)

10-20% volume/volume (V/V) fetal calf serum (FCS) (Gibco Invitrogen, UK)

100U/ml Pencillin-100µg/ml Streptomycin (Sigma-Aldrich, UK)

2mM L-glutamine (Gibco Invitrogen, UK)

2.1.3 Antibodies and Reagents

5D3 and 6C3 have been previously purified in our laboratory from supernatants from 5D3 and 6C3 hybridomas respectively, MR-derived constructs such as CTLD4-7Fc and CR-FN11CTLD1-3Fc were prepared in the lab by Dr. Martinez-Pomares as described (Martinez-Pomares et al., 2006).

GM-CSF was purchased from R&D System. Antibodies used for the phenotypic analysis of cells by FACS are shown in Table 2.3. Anti-ovalbumin rabbit polyclonal antibody (Lifespan biosciences) was used to confirm the presence of OVA by western blotting. Common secondary antibodies used for western blotting and ELISA were HRP and AP labelled donkey anti-mouse IgG (Jackson Immunoresearch laboratories) and goat anti-rat-IgG (CHEMI-CON). Antibodies used for the IL2 capture ELISA, rat anti-mouse IL-2 (Cat.

No. 554424), biotin rat anti-mouse IL-2 (Cat. No. 554426), and recombinant mouse IL-2 (Cat. No. 550069) used as ELISA standard, were purchased from BD Pharmingen, UK.

Sr. No.	*Product*	*Supplier*
1	Sensor Chip CM5 R4	GE healthcare Biosciences
2	HBS-EP Buffer	GE healthcare Biosciences
3	Acetate buffer pH4.0	GE healthcare Biosciences
4	Acetate buffer pH4.5	GE healthcare Biosciences
5	Acetate buffer pH5.0	GE healthcare Biosciences
6	Acetate buffer pH5.5	GE healthcare Biosciences

Table 2.1: Reagents and buffers used in the BIACORE studies.

TLR	*Agonist*	*Source/Supplier*
TLR4	Monophosphoryl Lipid A (MPLA) from *Salmonella minnesota* R595 (TLR grade)	Alexis Biochemicals, UK
TLR-9	CpG-B (ODN 1826)	Alexis Biochemicals, UK
TLR-4	Lipopolysaccharide (LPS)	Invivogen, UK

Table 2.2: Reagents used as innate stimuli in immunisation studies.

Antigen	*Conjugation*	*Clone*	*Isotype*	*Source*
CD206 (MR)	Alexa488	MR5D3	Rat IgG2a	Biolegend, UK
CD11b	PE/Cy7	M1/70	Rat IgG2b κ	BD Pahrmingen, UK
CD11c	PE	HL3	Armenian Hamster IgG1, λ1	BD Pahrmingen, UK
MHC-II	PE/Cy5	M5/114.15.2	Rat IgG2b, κ	Ebiosciences, UK
CD80	PE	16.10A1	Rat IgG2a	Ebiosciences, UK
CD86	PE	GL1	Armenian Hamster IgG	Ebiosciences, UK
CD24	PE	30.F1	Rat IgG	Abcam, UK
CD206 (MR)	unconjugated	MR5D3	Rat IgG2a	Prepared in our lab
CD3	Unconjugated	KT3	Rat IgG2a	Prepared in our lab
CD68 (Macrosialin)	Unconjugated	FA.11	Rat IgG2a	Prepared in our lab
Donkey anti-Rat	Alexa-Fluor488		Secondary Ab.	Invitrogen

Table 2.3: Antibodies (Abs) used for FACS staining and immunohistochemistry.

2.1.4 Buffers

1- Tris-saline Buffer
10 mM Tris-HCl (pH 7.4), 10mM $CaCl_2$, 154mM NaCl

2- Cell Lysis buffer
10 mM Tris-HCl, pH-8, 2% Triton X-100, 150 mM NaCl, 2 mM NaN_3, 2 mM Complete, Mini, EDTA-free inhibitor cocktail (Cat. No. 04693159001, Roche) 1 tablet per 5 ml of lysis buffer)

3- ELISA Washing Buffer
PBS supplemmented with 0.1-0.05% Tween 20 (Sigma)

4- ELISA Blocking Buffer (all other ELISA)
PBS supplemented with 3% BSA

5- ELISA Alkaline phophatase (AP) substrate buffer
Distilled water containing 100mM Tris-HCl pH 9.5, 100 mM NaCl, 1 mM $MgCl_2.6H_2O$.

6- ELISA Alkaline phophatase (AP) substrate solution
p-Nitrophenol phosphate tablets (Sigma) prepared at 1mg/ml in Alkaline phosphatase (AP) Substrate Buffer

7- TBS
10 mM Tris-HCl, pH 7.5, 10 mM Ca^{2+}, 154 mM NaCl and 0.05% (w/v) Tween 20

8- TBS-1M NaCl
10 mM Tris-HCl, pH 7.5, 10 mM Ca^{2+}, 1M NaCl and 0.05% (w/v) Tween 20

9- PBS
Prepare from tablets; 1 tablet per 100ml dH_2O (Oxoid Ltd. UK) or purchased from SIGMA

10- FACS Blocking Buffer
PBS, 5% v/v normal rabbit serum (Gibco), previously heat inactivated at 56 C for 30 mins. 0.5 % (w/v) BSA, 5mM EDTA, 2mM NaN_3

11- FACS Washing Buffer
PBS, 0.50 w/v BSA, 5mM EDTA, 2mM NaN3

12- FACS Fixing solution
PBS supplemented with 1% v/v formaldehyde (Sigma)

13- Ultra Pure Distilled water (DNase, RNase free)
Used for DNA/RNA work.

14- SDS-PAGE Running buffer
25 mM Tris, 90 mM glycine, 0.1% SDS (Biorad)

15- SDS-PAGE Loading Buffer (4X)
0.25 M Tris-HCl pH6.8, 8% SDS, 40% Glycerol, 0.1% Bromophenol blue, dH_2O

16- Western Blot Blocking buffer
PBS, 5% (w/v) non-fat milk powder, 0.1% Tween 20

17- Western Blot Wash Buffer
PBS supplemented with 0.1% Tween 20

18- Western Blot Transfer Buffer
25 mM Tris, 90 mM glycine, 20% methanol (Biorad)

2.2 General Methods

2.2.1 Protein expression and its downstream processing

Routine plasmid DNA isolation was done with the Qiagen mini prep kit following the manufacturer instructions but for transfection and protein expression large scale endotoxin-free plasmid isolation was done using the endotoxin-free plasmid DNA isolation kit (MACHEREY-NAGEL) following the protocol provided.

HEK-293T were grown in DMEM medium supplemented with FCS (10%), Glutamine (2mM), penicillin and streptomycin (100U/ml) until cells reach at 50-60% confluency. As HEK-293T are highly prone to sloughing off, changing the media and washing had to be done with great care. On the day of transfection media was replaced with 25ml of fresh complete DMEM media in the morning. In the afternoon endotoxin free plasmids (18μg) were mixed with Gene juice (Novagene), or home prepared Polyethylenimine (PEI) transfection reagent (54μl) in OPTI-MEM medium (1.8ml) and added onto the HEK-293T dropwise cultured in T-175 tissue culture flask. The day after transfection the

media was replaced with OPTI-MEM supplememnted with Glutamine (2mM), penicillin100U/ml-streptomycin 100μg/ml. After 4-5 days the supernantan was collected and centrifuged at 2100g for 15 mins two times. The supernatant was filtered using a 0.2μm filter (Ministart) and stored at 4°C. Protease inhibitors (Roche) were added in some instances.

2.2.1.1 Protein purification by Affinity Chromatography using Protein G and Protein A Sepharose

The antibodies and recombinant proteins in supernatants were purified by affinity chromatography on Protein G Sepharose (GE Healthcare, Amersham, UK), Protein A-Sepharose (GE Healthcare, Amersham, UK) or by using nickel column (GE Healthcare, Amersham, UK) depending on the nature of protein. Antibodies were eluted by using 0.1 M glycine (pH 2.8), and immediately neutralising with 1 M Tris-HCl (pH 8). The fractions collected were dialyzed against phosphate-buffered saline (PBS) using a dialyser cassette (Pierce, UK) in order to remove the low molecular weight contamination. Concentration of the purified Abs and other molecules was determined by BCA protein quantification assay which is based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of proteins (Pierce, UK). BCA Assay was performed according to the manufacturer's instructions by comparison with a standard curve made from BSA standards prepared in same diluents as the test samples.

2.2.1.2 Nickel affinity chromatography purification of His-tagged recombinant proteins

His-tagged proteins were purified using nickel-agarose columns (1 mL His-Trap HP column, GE Healthcare), exploiting the high affinity interaction between the nickel ion and the 6x histidine tag incorporated into the proteins. Protein was purified from 50 ml culture supernatant using an AKTA Prime apparatus. The column was first equilibrated in 5 column volumes of binding buffer (PBS containing 300 mM sodium chloride). The supernatant was loaded onto the column and the column was washed in buffer A (NaH_2PO_4 20mM, NaCl 300mM and Imidazole 10mM). The elution procedure used consisted in rising step-wise increments in the concentration of imidazole to reach to 500mM in PBS with 300mM sodium chloride. All components (flow through, starter and eluted materials) were collected. Eluted components were analysed by SDS-PAGE and fractions containing the required protein were collected and concentrated using ultra-15 centrifugal filter device (Millipore) with a 5 kDa molecular weight cut off.

2.2.2 SDS-PAGE

2-5μg of purified proteins samples were prepared in 1x SDS-PAGE loading buffer (0.12mM Tris-HCl, pH 6.8, 0.4%SDS, 5% glycerol, 0.02% bromophenol blue, dH_2O) containing 5% β-mercaptoethanol as reducing agent if required. The protein samples were heated at 100°C for 5 minutes and loaded into a 6-12% SDS-PAGE using Mini-PROTEAN III gel system (BioRad, USA). Electrophoresis was performed at 30 mA The gels were stained in Simply Blue safe Stain (Invitrogen) for one hour and destained in distilled

water for one hour according to the manufacturer's instructions. Gels were dried and stored using gel drying system (Invitrogen) according to the manufacturer's instructions.

2.2.3 Western Blotting

Gels were transferred to Hybond C membranes using a wet transfer apparatus over night at 200 mA. After transfer, filters were incubated in blocking buffer (5% non-fat milk in PBS-0.1% Tween 20) for one hour. After this the primary antibodies were added diluted blocking buffer and left to shake for an hour. After washes in blocking buffer secondary antibodies peroxidase conjugateddiluted in blocking buffer were added for 1 hour after which the filters were washed 3 times with PBS-0.1% Tween. The filters werer then incubated for one minute with ECL Western Blotting Detection Reagents (GE Healthcare) and dried with blotting paper. The filter was placed in the Kodak exposure box under a transparent sheet, and in the dark room the Kodak x-ray film is placed onto the transparent sheet and closed for a number of seconds depending on the exposure sought. The X-ray film was developed manually and hung to dry in an airing cupboard.

2.2.4 ELISA

2.2.4.1 ELISA

MAXISORP 96 well flat-bottomed plates (Nunc Cat no 439454) were coated with 5 μg/ml of antigen in PBS and incubated overnight at 4°C. The next day the plate was twice washed with PBS and blocked with 3% BSA in PBS for an hour at 37°C. It was then washed and incubated with primary antibodies diluted in PBS for 1 h. After washes secondary antibodies conjugated to alkaline phosphatase diluted in PBS were added for one hour. The plate was again washed , followed by 2 washes with AP developing buffer (100mM Tris-HCl, 100mM NaCl, 1mM $MgCl_2.6H_2O$ pH 9.5), before being incubated with 1mg/ml of p-nitrophenyl phosphate substrate (SIGMA) diluted in AP buffer. The OD absorbance was measured at 405nm on a 96-well plate reader (MultiskanEX; Labsystem) after incubating the plate for a few minutes at room temperature (3-5mins). All washes and incubations were carried out in 250μl PBS 0.1% (V/V) Tween-20 unless stated otherwise.

2.2.4.2 Lectin ELISA

The binding of different ligands to MR-constructs was analysed by Lectin ELISA. MAXISORP 96 well flat bottomed plates were coated with 50 μl of relevant ligand or control sugars at required concentration diluted in PBS and sealed in parafilm before being incubated overnight at 4°C. The next day the plates were washed 3 times with TBS (10 mM Tris-HCl, pH 7.5, 10 mM Ca^{2+}, 154 mM NaCl and 0.05% (w/v) Tween-20) and incubated with 5 μg/ml of MR constructs (CTLD4-7-Fc or CR-FNII-CTLD1-3Fc) for 1.5-2 hours at room

temperature. Then, they were washed 3 times with TBS and incubated with a 1:1000 dilution of anti-human IgG Fc-specific, alkaline phosphatase conjugate in TBS (SIGMA/Jackson Laboratories) for an hour. The plates were again washed 3 times with TBS, followed by 2 times with AP developing buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2.6H_2O$ pH 9.5), before being incubated with 1mg/ml of p-nitrophenyl phosphate substrate (SIGMA). The OD absorbance was measured at 405nm on a 96-well plate reader (MultiskanEX; Labsystem) after incubating them for couple of minutes (3-5mins). Note that all washes were carried out in 250µl TBS unless stated otherwise. Incubations were performed in 50 µl and development in 100 µl.

2.2.4.3 Inhibition ELISA

To confirm the specificity of the binding of MR constructs, 2 µg/ml solutions of MR constructs were prepared in TBS-1M NaCl with or without the competing sugars (D-galactose or D-mannose 50 mM) and incubated for 30 min at room temparature. These solutions were then used for the incubation with the test ligands. The rest of the procedure was performed as described in the previous section.

2.2.4.4 IL-2 Capture ELISA

A 96-well microtitre plate was coated with capture monoclonal antibody rat antimouse IL-2 (PharMingen; Cat: 554424) 1:500 diluted in PBS, overnight at 4°C. A blocking buffer (PBS, 10% FCS, 0.1% NaN_3) was then added after washing 3 times with PBS-0.05% Tween-20 followed by 45 min incubation at 37°C. Again, the plate was washed 3 times in washing buffer (PBS

supplemented with 0.05% Tween -20) before transferring 50µl of supernatant or recombinant mouse IL-2 (cat: no. 550069) standard diluted in R10 media to appropriate wells and left at room temperature for 2 hours. After that wells were washed 4 times with PBS/Tween, and biotinylated secondary mAb rat antimouse IL-2 (PharMingen; Cat: 554426) was added which was made at 1:400 diluted in blocking buffer. After 1 hour, wells were washed with PBS/Tween, and a 1:1000 dilution of ExtrAvidin-AP (Sigma; Cat: E3626) was added for another hour, washed thoroughly 4 times with PBS/Tween, and 2 times with AP developing buffer before p-nitrophenyl phosphate substrate (SIGMA) was added into each wells. The OD value absorbance was read at 405nm.

2.2.5 Endocytosis assay

In order to analyze MR mediated internalization capacity Chinese Hamster Ovary (CHO) cells expressing MR (Martinez-Pomares et al., 2003) were used. The CHO-MR and CHO cell lines were grown in 24-well plates in F12 media (Invitrogen) containing 10% FBS and 100U/ml Pencillin-100µg/ml Streptomycin. Once the cells were ready reached desired density the media was removed from the wells and 1ml of the F-12 with Glutamax and100U/ml Pencillin-100µg/ml Streptomycin (P/S) was added to remove the serum and cells were incubated for 30 minutes at 37 °C. The plate was washed with PBS. Next, test ligands were added diluted in RPMI 1640 containing P/S. After 30 minutes incubation, cells were fixed with 2% formaldehyde in PBS on ice for 20 minutes, washed with PBS (1ml per well) and permeabilized and blocked by incubation with 0.1% saponin in blocking buffer (5% heat-inactivated rabbit

serum; 0.5% BSA; 2mM NaN_3; 5mM EDTA in PBS) for 30 minutes. To detect uptake of rat anti-MR antibodies cells were incubated with anti-rat IgG-Alexa-488 on ice for one hour followed by three washes with washing buffer (0.5% BSA; 2 mM NaN_3; 5 mM EDTA in PBS) containing 0.1% saponin. Then, 250µl of 2mM Trypsin-EDTA solutions were added into each well and left for few minutes. Cells were detached and transferred to FACS tubes containing 250µl of 2% formaldehyde. Samples were analysed on a Cytomics FC500 (BeckmanCoulter), flow cytometer and data was analysed using WEASEL software.

2.2.6 Labelling and flow cytometric analysis of cells

Supernatants were removed and cells were incubated with 50 ul of blocking buffer containing 10ug/ml of 2.4G2 (Anti-Fc receptor Ab) on ice for 30 minutes. Then they were mixed with 50ul of 20ug/ml of labelled antibody in FACS blocking buffer and kept on ice for an hour. Cells were washed 3 times with FACS Wash (0.5% BSA; 2 mM NaN_3; 5 mM EDTA in PBS). They were transferred into FACS tubes containing 250 ul of 2% formaldehyde in PBS. Flow cytometry was performed on a CytomicsFC500 (BeckmanCoulter) and data were analysed with WEASEL software. The cells were labelled with following antibodies along with their isotype controls; anti-MR (5D3) labelled with Alexa-488, anti-MHC class II of PECy5 labelled, anti-CDIIc of PE labelled and anti-Dectin-1 FITC-labelled.

2.3 Culturing of primary cells

Tissue culture plastic was purchased from BD Biosciences (UK), BDH (UK), Corning Corporation (USA) and Fisher scientific (UK).

2.3.1 Harvesting mouse bone marrow and generation of GM-CSF induced DC (BM-DC).

After removing all muscles and rotulas from the femurs and tibias with clean tissue, the bones were placed into a petri dish containing 70% ethanol for a minute to sterilise the bones. Then they were washed 3 times with RPMI+P/S medium. Both ends of the bones were cut with sterilised scissors and the cut bones were placed in a clean petri dish and the bone marrow was flushed out using 10 ml syringes filled with RPMI medium 1640 supplemented with 10% FBS (SIGMA-Aldrich), 2mM L-glutamine, 100 units/ml Penicillin-100 units/ml streptomycin. 21G needles were used for femurs and 25G for tibias. The bone marrow was centrifuged at 235g, 4°C for 5 min. Supernatant was removed and the cells were resuspended in ~10ml of R10 medium containing GM-CSF 1000 U/ml (R&D systems catalog no.415-ML) while harshly pipetting in and out to break up the bone marrow. The cells then were counted before being plated in media in the presence of GM-CSF 1000 U/ml at density of no more than $4x10^5$cells/ml in 100 mmx20mm TC plates (Corning). Cells were placed in incubator at 37°C/ 5% CO_2 and at day 3, the media was gently removed with vacuum by holding culture dish at shallow angle after gentle swirling, leaving enough media in the dish to cover the bottom so that the cells do not become dry. Media containing GM-CSF 1000U/ml was replenished

afterwards before leaving them in the incubator. The same wash was repeated on day-6 and BMDCs were collected at day 7.

2.3.2 Generation of bone marrow derived macrophages

Bone marrow was collected according to the protocol described in section 2.3.1 and resupended in 25 ml of RPMI medium 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml Penicillin, 100µg/ml streptomycin and 15% L-conditioned media (LCM) added to a 15O mm petri dish. On day 3, 15 ml of RPMI complete media was added into each petri dish. On day 6 the old media was replaced with the fresh 25 ml RPMI complete media containing LCM. Macrophages were collected by treating the cells with cold PBS containing 10mM EDTA. Macrophages were resuspended in RPMI complete media and plated for further use.

2.3.3 Generation of monocyte derived human dendritic cells

The blood was mixed with PBS or Hank's solution at a 1:1 ratio. The mix was layered on 5ml of histopaque (Histopaque-1077, Sigma-Aldrich, UK) using a Pasteur pipette and centrifuged at 800 g for 23 minutes (acceleration 1, de-acceleration 0). After centrifugation, peripheral blood mononuclear cells (PBMC) were collected using Pasteur pipette, diluted with 30 to 40 ml of Hank's and centrifuged again and resuspended in 10ml of Hanks followed by cell counting. Cells (10^7) were incubated with mouse anti-human CD14 mAb conjugated to magnetic beads (20 µl) in 80 µl MACS buffer (Miltenyi Biotec, UK) for 20 min at 4°C,. The cells were washed with 1x MACS buffer and loaded onto MS-Column in the presenc of a magnetic field; the column was washed three times with 50µl MACS buffer and removed from the magnetic

field and cells were collected into a new tube with 2 ml MACS buffer by applying a plunger. The cells were washed with RPMI medium and resuspended in DC-medium (RPMI + 10% FCS) supplemented with IL-4 (250 IU/ml) and human GM-CSF. Fresh DC-media supplemented with human IL-4 and human GM-CSF (50 ng/ml) (R&D Systems) was added at day 3. Dendritic cells were used at day 6.

3 Generation of chimeric anti-MR Ab by genetic engineering

3.1 Introduction

DCs are professional APC that have much scope for the development of vaccines through different immunotherapeutic approaches. Synthetic peptides or antigens have a very short half life and do not induce strong immune responses as they don't specifically target the APCs. Therefore different strategies are adopted; including immunisation with DCs pulsed with appropriate Ag, naked DNA using gene gun methodology and mixtures of Ags and adjuvants (Bellone et al., 2000). A new trend in the immunotherapy is to deliver protein Ags to the APC using receptor-specific Abs. Targeting Ag through antibodies against DC receptors is the ideal technique due to their specificity and higher affinity (Keler et al., 2007). Receptors used in Ag targeting mostly belong to the C-type lectin receptor (CLR) family, which are calcium-dependent lectins and share primary structural homology in their carbohydrate recognition domain (CRD). The common CLRs used in targeting studies are the mannose receptor (MR, CD206), CD205 and DC-specific intercellular adhesion molecule-3 (ICAM3)-grabbing non-integrin (DC-SIGN). Ag targeting through different receptors might result in different immune outcomes due to different intracellular receptor routing, signalling pathways and expression patterns (Tacken et al., 2007).

Targeting of antigen through MR using anti-MR antibody (Ramakrishna et al., 2004, He et al., 2007, Tsuji et al., 2010) or glycosylated Ags such as the model antigen ovalbumin (OVA) results in cross presentation and activation of cytotoxic T-cell responses. This fascinating finding makes MR a strong candidate for the development of therapeutic vaccines against infections that

require T-cell-mediated immunity such as viral infections and tumours (Burgdorf et al., 2008). MR is an efficient endocytic receptor expressed by murine bone-marrow derived DCs and its APC subtypes present in mice (Linehan et al., 2001, McKenzie et al., 2007). MR specific antibodies fused cancer antigen NY-ESO-1 have been used to target dendritic cells specifically and result in the induction of antigen specific $CD8^+$ and $CD4^+$ T-cells. DCs targeting through MR in the presence of an appropriate adjuvant leads to corss-presentation of Ag and induce of powerful CTL immunity to facilitate immunotherapy of cancer (Tsuji et al., 2010).

Martinez-Pomares and Reid et al. described the first panel of mAb against murine MR and have characterised several mAb with distinct binding abilities (Martinez-Pomares et al., 2003). While the anti-MR mAb 6C3 targets efficiently to MR^+DC *in vivo*, investigation into the humoral responses elicited in response to anti-MR mAb demonstrated that another anti-MR specific Ab, with a higher affinity for MR, 5D3, elicited a more robust response than 6C3 even though both Ab were raised against the same region of the receptor (McKenzie et al., 2007). These results suggest that increased ability to bind to MR (as shown for 5D3) could lead to enhanced T-cell responses. Based on these observations we propose that one of the factors that could influence how MR-targeted Ag is presented to the immune system is the strength of the ligand-receptor interaction. Based on these observations and other studies showing antigen targeting through MR leads to cross-presentation, we aimed to generate chimeric anti-MR antibodies carrying model antigens.

The present chapter describes the generation of chimeric anti-MR mAb (5D3-HEL) bearing a mutated Fc portion of mouse IgG1 and a hen egg lysozyme (HEL) derived epitope. Plasmid constructs carrying anti-DEC205 Ab-HEL fused to a mutated Fc region of mouse IgG1 (Clynes et al., 2000), kindly provided by Prof Nussenzweig (Rockefeller University, New York) were used as starting materials. Binding properties of the anti-MR mAbs 5D3 and 6C3 were compared using BIACORE. The variable regions; VH and VL of 5D3 were cloned and used to replace the variable region of DEC205-HEL to obtain 5D3-HEL. This chapter also describes expression of DEC205-HEL, 5D3-HEL and the control protein IgG1-HEL, and their initial characterisation in terms of their binding to DEC205 and MR respectively.

3.2 Materials and methods

3.2.1 Characterization of starting materials: 5D3 and 6C3 anti-MR monoclonal antibodies

3.2.1.1 SDS-Polyacrylamide gel electrophoresis (PAGE)

5-10μg of purified anti-MR mAbs were analysed using SDS-PAGE by following the protocol described in section 2.2.2.

3.2.1.2 Two-dimensional gel electrophoresis and Q-TOF sequence analysis of spots corresponding to light chains

Purified mAbs (5D3 and 6C3) (30-50μg) were dissolved in rehydration buffer (Lysis buffer) containing 7M urea, 2M thiourea, 4% CHAPS (w/v), 10mM DDT, 0.5% (w/v) carrier ampholyte (pH3-10) and 0.002% bromophenol blue

(w/v). The strips loading tray was washed with 10% SDS and rinsed with distilled water. The samples were loaded on the tray and 17cm pH3-10 IPG ready strips were placed over it carefully, ensuring no bubbles were formed under the lid. The tray was placed in a Biorad Protean isoelectric focussing (IEF) Cell and was set to passive rehydration mode at 20°C overnight. After rehydration the strips were transferred into the IEF cell and wet electrode wicks were placed underneath each strip. The conditions used for IEF were: 250V for 15 min, 10,000V for 3 h, 70,000 V for approximately 12 h and hold at 500V at 20°C. After IEF, IPG ready strips were washed three times on the shaker for 15 min in equilibration buffer-I, containing 50 mM Tris-HCl, 6 M urea, 29.3% (v/v) glycerol, 2.0% (w/v) SDS, 0.002% (w/v) bromophenol blue and 1% (w/v) dithiothreitol (pH 8.8). This was followed by an additional 15 min wash in the same buffer with 2.5% (w/v) iodoacetamide in place of dithiothreitol. After equilibration, the IPG strips were sealed on top of 12% SDS-PAGE gels with 0.5% (w/v) agarose containing 0.002% (w/v) bromophenol blue. The SDS-PAGE was run at a constant current of 60 mA for 4 h, using Protean-II Gel electrophoresis system (Biorad). The gels were stained with Simply Blue safe stain (Invitogen) and scanned onto a densitometer. Images were analyzed using PD Quest and spots of interest were cut from the gel for identification by Q-T0F sequencing at the Biopolymer Synthesis and Sequencing Facility, University of Nottingham.

3.2.1.3 Analysis of binding properties of 5D3 and 6C3 by surface plasmon resonance (SPR)

Binding properties of the Anti-MR mAbs, 5D3 and 6C3 were compared by surface plasmon resonance (SPR) using BIAcoreX (BAIcore AB). CM5 research grade biosensor chips, *N* hydroxysuccinimide (NHS), *N*-ethyl-*N*0-(3-diethylaminopropyl) carbodiimide (EDC), ethanolamine-HCl, and HBS-EP buffer were obtained from (GE Healthcare, UK). Recombinant ligands CTLD4-7-Fc and CR-FNII-CTLD1-3 were expressed in 293T-cells and purified by affinity chromatography on Protein A Sepharose (GE Healthcare) in our lab as described in Material and Methods (Linehan et al., 2001) and used to assess MR specific binding of anti-MR mAbs. Two channels of a research-grade carboxymethyl dextran chip (CM5) were activated with NHS/EDC for 5 min to give reactive succinimimed esters and to facilitate the conjugation of ligands using an amine coupling procedure as described previously (Johnsson et al., 1991). Recombinant ligands CTLD4-7-Fc and CR-FNII-CTLD1-3-Fc were diluted in sodium acetate buffers with different pH (pH5.5, pH5, pH4.5, pH4) to a final concentration of 30μg/ml in each sample in order to find out their appropriate immobilisation pH. The ligands prepared at pH 4.5 were injected until 10,000 resonance units (RU) of protein were coupled. Remaining activated groups were then blocked with a 5-min injection of 1M ethanolamine (pH 8.5). After this, purified 5D3 and 6C3 at a range of concentrations (1.5-3μM) were injected over the chip in order to determine their binding capacity to the immobilised ligands. The dissociation phase was then monitored for 900 s, followed by a 50 μl injection of 10mM glycine, pH 2.0, to regenerate a fully active chip by removing the bound antibodies. All kinetic experiments were

performed at 25 °C in a buffer containing 10mM Hepes, pH 7.4, 150mM NaCl, and 0.005% Tween -20, at a flow rate of 20µl/min. In control experiment, CR-FNII CTLD1-3-Fc was used instead of CTLD4-7-Fc to evaluate the non-specific binding of 5D3 and 6C3, and rat IgG2a was used to assess non specific binding of antibodies. To remove contributions of non-specific binding and refractive index, the control responses were subtracted from those obtained from the active surface. The kinetics parameters such as the rate of association and dissociation were calculated using BIA evaluation software 3.1 (Biacore AB) (Myszka and Morton, 1998).

3.2.2 Characterization of the DEC205-HEL and IgG1-HEL control

3.2.2.1 Plasmids constructs

The plasmid constructs encoding for the variable region of the control rat IgG2a (IgG1-HEL) and DEC205 (DEC205-HEL) fused to a mutated Fc region of mouse IgG1 unable to bind Fc receptors (Hawiger et al., 2001) were provided by Prof. M. Nussenzweig (Rockefeller University). A DNA fragment encoding the HEL peptide 46-61 was present at the C-terminus of the heavy chains of these plasmid constructs. The DEC205-HEL vectors (Figure 3.1) contain the restriction sites EcoRI and Not-I on both sides of the gene and have the ampicillin resistance gene for the selection of transformed bacteria but the exact nature of the expression vector was unknown.

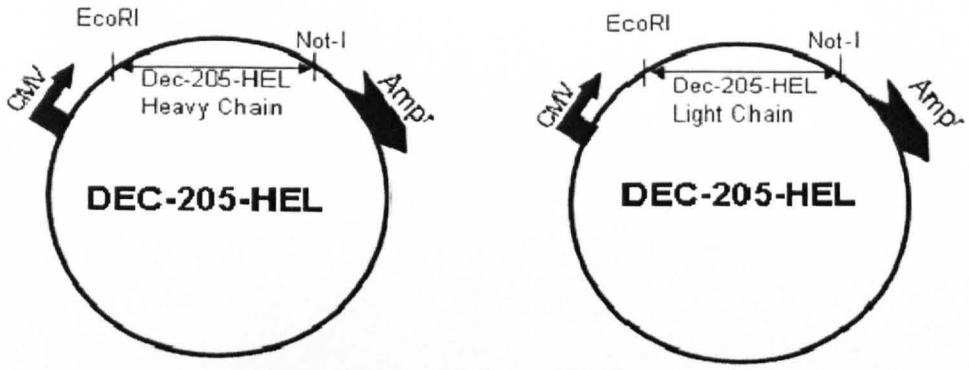


Figure 3.1: DEC205-HEL vectors carrying the heavy and light chains of the anti-mouse DEC205-HEL antibody

3.2.2.2 Plasmid DNA isolation

Routine plasmid DNA isolation was done using the Qiagen mini prep kit according to the manufacturer's instructions. Endotoxin-free plasmid isolation was done using the endotoxin-free plasmid DNA isolation kit (MACHEREY-NAGEL) following the protocol provided.

3.2.2.3 Transformation

Plasmids were transformed in the high efficiency DH5α competenT-cells or Top10 (Invitrogen) according to the manufacturer's instructions. The transformed cells were grown overnight in LB Agar (SIGMA) in the presence of ampicillin sodium salt (Sigma) (100μg/ml, Sigma) with X-gal, (40μg/ml, Roche) and IPTG (0.1mM, Roche) when required.

3.2.2.4 Restriction digestion

Roche restriction enzymes (EcoRI, NotI, HpaI, ApaI and PstI) were used in this work according to the manufacturer's instructions. Reactions were incubated at 37°C for 2-4 hours.

3.2.2.5 DNA Gel electrophoresis

DNA samples were analysed using Sybersafe-stained 1-2% (w/v) agarose (Invitrogen) gel in Tris-Acetate-EDTA (TAE) buffer (4.8 gm/l Tris base, 1.14 gm/l acetic acid and m1M EDTA, pH 8.5) and analysed under the UV by Gel documentation and an imaging system. For the retrieval of DNA for cloning purposes Blue Light, Dark reader transilluminator (Clare Chemical Research) was used. In some instances gels were stained using Ethidium Bromide (10 ng/ml).

3.2.2.6 Gel extraction

DNA was extracted from gels using the Qiagen DNA extraction kit according to the manufacturer's instructions.

3.2.2.7 Ligation reaction

DNA was ligated with the help of T4 DNA ligase (Roche) using a 3:1 molar ratio of insert to vector in a final volume of 10µl and incubated on ice overnight allowing the ice to thaw.

3.2.2.8 Sequencing of the vector carrying DEC205-HEL

Primers were designed on the basis of the known heavy chain sequence (Appendix-1) and obtained from MWG-Biotech. A partial sequence of the vector was obtained using the HR-1 and HF-1 primers in the Biopolymer synthesis and analysis unit, Nottingham University. The sequences of the primers are given in Table 3.1.

Primer name	Primer sequence	GC content (%)	Tm (°C)
HR-1	AACCACTCAGGTGCCTGC	61.1	58.2
HF-1	CCATACTGAGAAGAGCCT	50.0	53.7

Table 3.1: Forward (HF-1) and reverse primers (HR-1) used for the sequencing of the vector carrying DEC205-HEL.

3.2.2.9 Expression of DEC205-HEL and IgG2a in HEK-293T

DEC205-HEL and IgG2a-HEL antibodies were expressed in HEK-293T by following the protocol as described in section 2.2.1.

3.2.2.10 Purification of DEC205-HEL and IgG1-HEL antibodies by affinity chromatography

The supernatant collected from transfected cells was purified by affinity chromatography on Protein G Sepharose Gamma bind plus (GE Healthcare, Amersham, UK) as described in section 2.2.1.1. Containers used in protein purification such as beakers and tubings were washed in trigene, 0.5 M NaOH and rinsed in water and PBS to avoid LPS contamination

3.2.2.11 SDS Polyacrylamide gel electrophoresis (PAGE)

5-10μg of purified DEC205-HEL and IgG2a-HEL were mixed with SDS-PAGE loading buffer containing 5% β-mercaptoethanol as reducing agent and resolved on a 12% SDS-PAGE by following the protocol described in section 2.2.2.

3.2.2.12 Preparation of thymus lysate and its analysis by western blotting

In order to prepare thymus lysate, mouse thymus was collected and sliced into pieces using a scalpel. The tissue was homogenized using mortor and pistle in the presence of lysis buffer (10mM Tris-HCl, pH 8, 2% Triton X-100, and 150 mM NaCl, 2mM NaN3, 2mM EDTA) supplemented with protease inhibitors (Roche). This grounded tissue was centrifuged to eliminate the debris and insoluble material and soluble protein was quantified by BCA quantification method. 15-20μg of thymus lysate was loaded onto 6 % SDS-PAGE gels. Gel electrophoresis was performed at 30 mA per gel; after all the samples had run through the gel, they were transferred to Hybond C (GE, Healthcare, UK) membranes using a wet transfer apparatus overnight at 200mA.

Filters were incubated in blocking buffer (5% non-fat milk in PBS 0.1% Tween- 20) for one hour. Primary antibodies (DEC205-HEL and IgG1-HEL) were then added at 2μg/ml in blocking buffer and left to shake for an hour. After washes the secondary antibody, donkey anti-mouse IgG peroxidase conjugated (Jackson laboratories) [diluted 1:2000 dilution in Blocking buffer] was used for 1 hour and films were developed by following the protocol described in section 2.2.3.

3.2.3 Amplification of the variable region of 5D3 monoclonal antibody using 5'-RACE

3.2.3.1 Hybridoma culture and RNA extraction

5D3 hybridoma cells were cultured in IMDM complete medium containing 20% FCS. 5×10^6 to 10×10^6 hybridoma cells were used for RNA isolation. The

cells were centrifuged for 10 min at 233 g. The supernatant was discarded and the cells were washed with PBS. Total RNA was isolated using the A&B gene RNA isolation kit or the RNeasy Mini Kit (QIAGEN), according to the manufacturer's protocols.

3.2.3.2 cDNA synthesis

cDNA synthesis was done according to the manufacturer's instructions provided with the 5'RACE, Clontech kit using 5'CDS primer A mix (random primers) and the MMLV reverse transcriptase (Invitrogen).

3.2.3.3 Amplification of variable genes by 5'RACE

5' primers were designed against the conserved regions of the heavy and light chain of rat immunoglobulin (IgG 2a kappa) for the 5'RACE in order to get the variable regions of the heavy and light chains of 5D3. These primers were obtained from MWG Biotech; their sequences are shown in Table 3.2.

Primer name	Primer sequence	GC content (%)	Tm (°C)
VHC-1	TGACGGTCTCGCTGGGCCAGGTG CTG	69.2	72.7
VKC-1	GAAGGAACTGGGGTGCAGGTGG CAC	64.0	69.5

Table 3.2: Primers designed against the conserved regions of the heavy (VHC-1) and light chains (VKC-1) for 5'RACE and amplification of the variable regions of 5D3.

5'RACE ready cDNA was used for the 5'RACE of the heavy and light chains using Clontech, "SMART™ RACE cDNA Amplification Kit" and Adanvantage2 PCR enzyme system (Clontech), according to the following PCR programme:

5 cycles: 94°C 30 sec; 72°C 3 min
5 cycles: 94°C 30 sec; 70°C 30 sec; 72°C 3 min
25 cycles: 94°C 30 sec; 68°C 30 sec; 72°C 3 min

5' RACE products were analysed by agarose gel electrophoresis (1% w/v) and were extracted from the gel using the gel extraction kit provided with the SMART RACE cDNA amplification kit, or using QIAGEN gel extraction kit, according to the manufacturer's instructions. This extracted DNA was cloned into the 2.1 TOPO cloning vector provided with the TOPO TA cloning kit (Invitrogen, Figure 3.2) and transformed into One Shot E.*coli* cells. The transformed cells were incubated overnight at 37°C on LB agar plates containing 100 μg/ml ampicilln, 0.1mM IPTG and 40μg/ml X-Gal. White colonies were selected and screened by restriction digestion with EcoRI and colony PCR using M13 forward primer and M13 reverse primers. Positive clones were sequenced at Biopolymer Synthesis and Analysis unit of Nottingham University.

3.2.3.4 DNA Sequence analysis

Sequences obtained were analysed using MEGA 4.1 and SEQ-MAN software. DNA contigs of the clones were made by equating the forward and reverse sequences of the clones. DNA contigs of the clones were translated with MEGA 4.1 and nucleotide sequences of light and heavy chains were further analysed by IMGT V-Quest software **(Ruiz et al., 2000).**

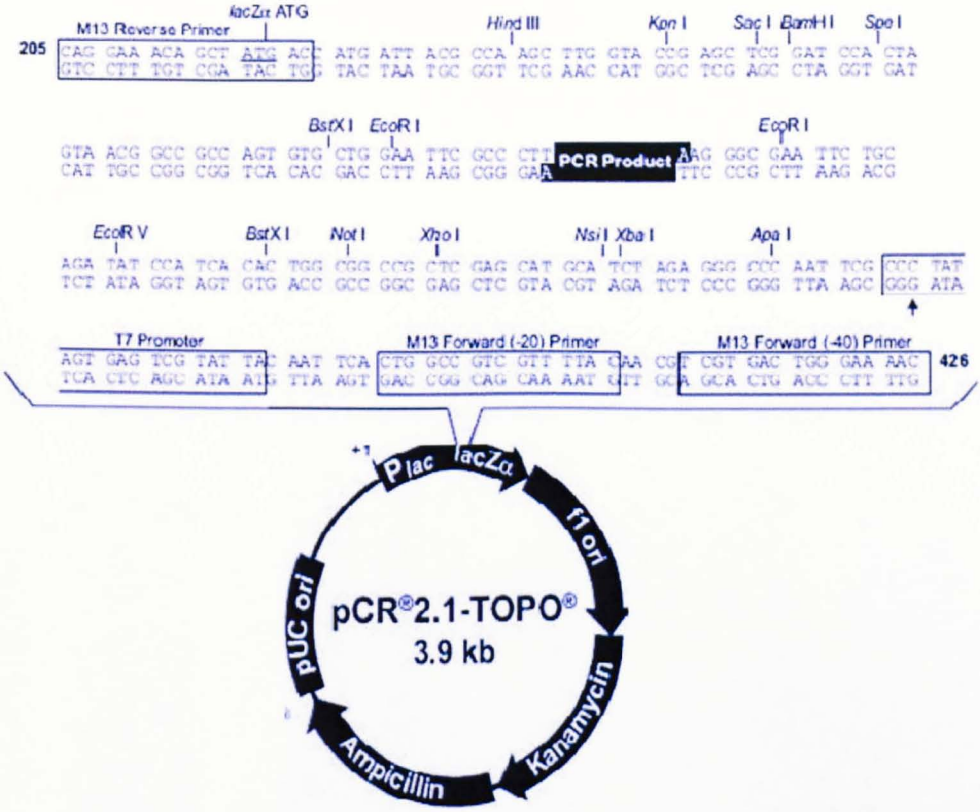


Figure 3.2: The map shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site

3.2.3.5 Replacement of the variable region of anti-DEC205 (Heavy and light chain) with the variable region of 5D3 by restriction cloning

The variable region of DEC205-HEL was replaced with the variable region of 5D3 Ab by restriction cloning. Restriction sites in the constant regions of the heavy and light chains were predicted by using the 'Gene Tool Lite' software. Restriction digestion was performed with the selected restriction enzymes in order to confirm that they would cut only one specific site in the whole plasmid.

Restriction primers were designed based on the DNA sequence of the heavy and light chains of the 5D3 and DEC205-HEL antibodies.

Primer name	Primer sequence	GC content (%)	Tm (°C)
FH (EcoR1)	TATAGAATTCTGCCATGGACAGGCTTA CTTCCT	42.4	67.0
RH (ApaI)	ATATGGGCCCGTTGTTTCAGCTGAAGA CAGT	48.5	69.5
FL (EcoR1)	TATAGAATTCCACCATGGCCATGAAGA CGCCTGC	50.5	70.7
RL (HpaI)	ATATGTTAACTGCTCACTGGATGGTGG GAAGATGGATAC	43.6	70.5

Table 3.3: FH (EcoRI) and RH (ApaIA) are the forward and reverse primers for the heavy chain; FL (EcoRI) and RL (HpaI) are the forward and reverse primers for the light chain. Restriction sites are highlighted

Clones carrying the variable regions of the heavy and light chains of 5D3 were used as template and amplified with Advantage 2PCR kit (Clontech) on 'Primus 96 advances Gradient Thermocycler' (peQLab) by adopting the following cycling parameters: 95°C for 1 min and 25-35 cycles; 95°C for 30 sec, 68°C for 1 min, and 68°C for 1 min. PCR products carrying the relevant restriction sites and plasmids were digested with the same set of enzymes and purified by gel extraction kit (QIAGEN). The products were ligated into the pre-digested DEC205-HEL vector using T4 DNA ligase (Roche) as described in section 3.2.2.7. Ligated clones are transformed into TOP-10 chemically competent E.coli and screened by colony PCR, restriction digestion and sequencing using the DEC-205-HEL vector specific primers as shown in Table 3.4.

Primer name	Primer sequence	GC content (%)	Tm (°C)
MV-F-Primer	CACATACGATTTAGGTGACAC	42.9	55.9
MV-F2-Primer	GGATCCCTGTCCAGCGGTGTGC	68.2	67.7
MV-R-Primer	GGTGCTGCGCGAATTAATTCCCGATCC	55.6	68.0

Table 3.4: The product cloned into the DEC205-HEL vector was sequenced by using vector specific primers such as MV-F and MV-R-primers. . MV-F2-Primer

was designed complementary to the constant region of the heavy chain to facilitate the sequencing of long strand of heavy chain insert.

3.2.4 Expression of anti-MR chimeric antibody carrying HEL antigen (5D3-HEL) and analysis of its bining properties using ELISA

Chimeric antibodies (5D3-HEL) and isotype control (IgG1-HEL) were texpressed in HEK-293T. Antibody purification was performed as described in Section 2.2.1. Expression of the antibodies was confirmed by Western Blotting using donkey anti-mouse IgG conjugated to peroxidase according to the protocol described in Section 2.2.3.

The binding of 5D3, 5D3-HEL (chimeric antibody) and their isotype controls was analysed by ELISA. MAXISORP 96 well flat-bottomed plates (Nunc Cat no 439454) were coated with 5μg/ml of chimeric antibodies. The coated plates were incubated overnight at 4°C. The next day the plate was twice washed with PBS and blocked with 3% BSA in PBS for an hour at 37°C. It was then washed with PBS 0.1% (v/v) Tween-20 and incubated with 1:1000 dilutions of secondary antibodies: anti-rat IgG-AP (SIGMA) and anti-mouse IgG-AP (SIGMA) respectively for one hour. The plate was again washed 3 times with PBS 0.1% (V/V) Tween-20, followed twice with AP developing buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2.6H_2O$ pH 9.5), before being incubated with 1mg/ml of p-nitrophenyl phosphate substrate (SIGMA) in AP buffer. The OD was measured at 405nm on a 96-well plate reader (MultiskanEX; Labsystem) after incubating for a few minutes (3-5mins). All washes and incubations were carried out in 250μl PBS 0.1% (V/V) Tween-20 unless stated otherwise.

3.3 Results

3.3.1 Characterisation of anti-MR mAbs 5D3 and 6C3

3.3.1.1 Analysis of the binding properties of 5D3 and 6C3 using Surface Plasmon Resonance (SPR)

We hypothesised that the strength of interaction between MR and anti-MR mAb could influences Ag processing and T-cell differentiation. The binding pattern of two anti-MR mAbs (5D3 and 6C3) was analysed and compared using BIAcore, to quantitatively study their interaction with MR. In order to immobilise the required reactive units of the ligands on the chip, the binding of ligands CTLD4-7-Fc and CR-FNII-CTLD1-3-Fc (Martinez-Pomares et al., 2006, Zamze et al., 2002) to the chip surface was analysed at different pHs (pH5.5, pH5, pH4.5, pH4). Optimal binding of the recombinant ligands to the chip was observed at pH 4.5 (Figure 3.3). Once MR ligands were immobilised onto the two channels of the biosensor chip, 5D3 and 6C3 mABs were passed over them at different concentrations (in the range 0.25μM – 3.0μM) to analyse their binding characteristics. The association rate of 6C3 (1.61e-4) was found higher than that of 5D3 (1.1e-4); however, the dissociation rate of 6C3 (2.27e3) was also higher than 5D3 (1.83e3), indicating that the interaction of 5D3 with MR (CTLD4-7 Fc) is more stable than 6C3 (Table 3.5).

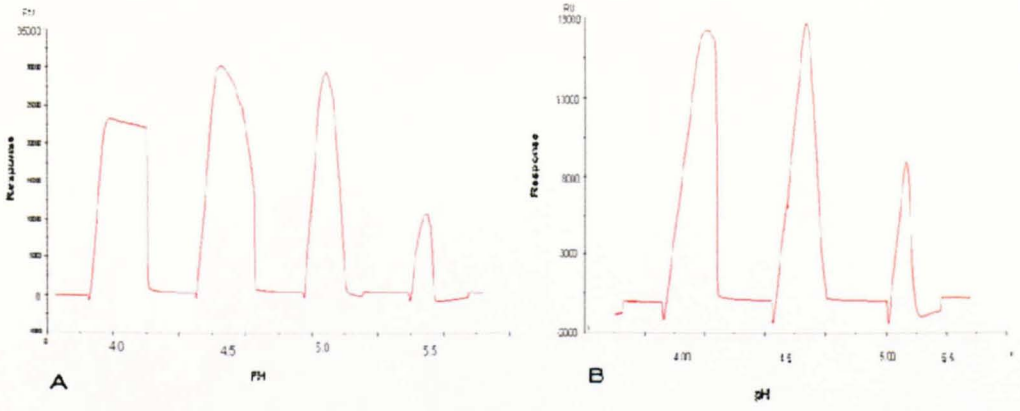


Figure 3.3: pH-dependency of the coupling of CTLD4-7-Fc and CR-FNII-CTLD1-3Fc to the sensor chip.

Sensograms show the binding of recombinant ligands (A) CTLD4-7-Fc and (B) CR-FRII-CTLD1-3-Fc via amine coupling to the chip at pH 4.0, pH 4.5, pH 5.0 and pH 5.4

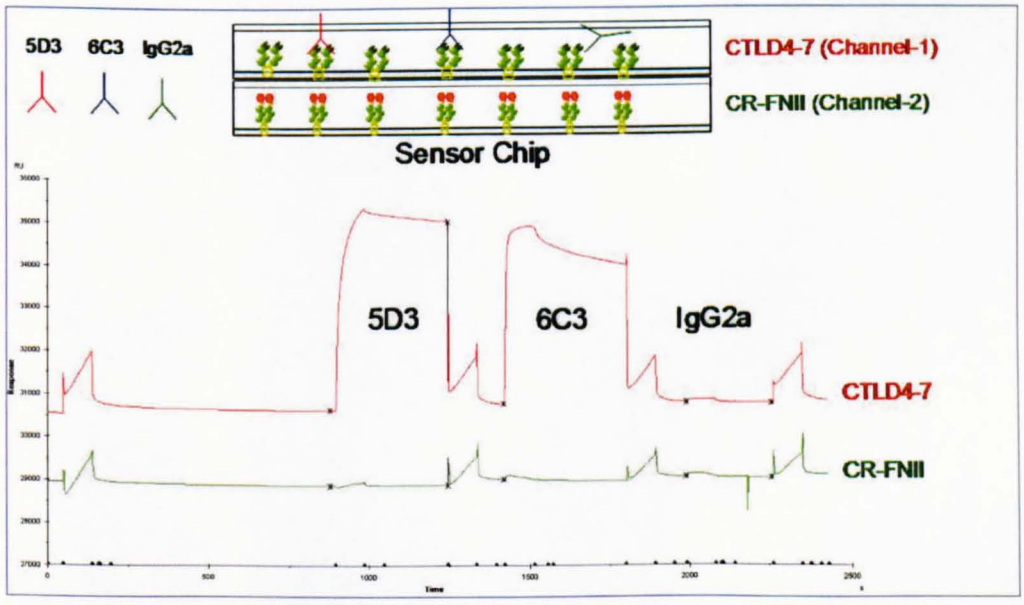


Figure 3.4: Specific recognition of CTLD4-7-Fc by anti-MR mAb 5D3 and 6C3.

Sensor chip was coated with MR ligands such CTLD4-7-Fc and CR-FNII in channel-1 and channel-2 respectively. Curves shown in red line indicate the binding of 5D3, 6C3 and IgG2a with CTLD4-7-Fc and in green it shows their binding with CR-FNII, used as a negative control.

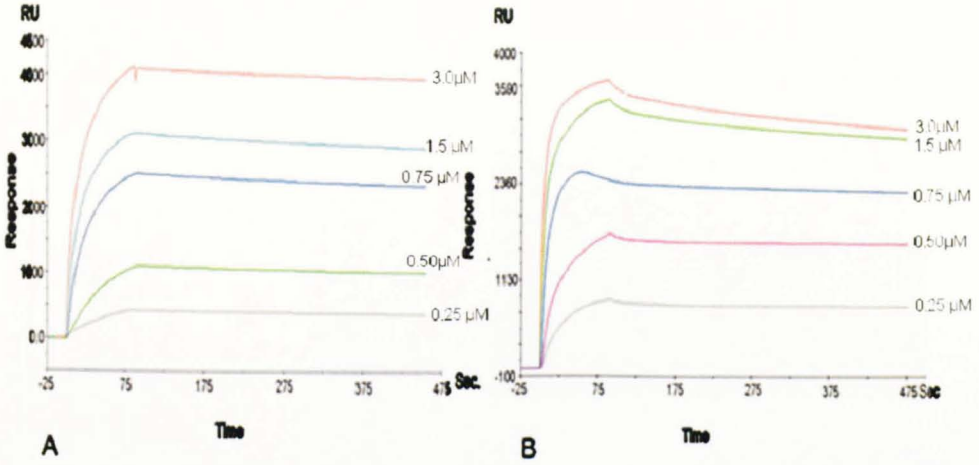


Figure 3.5: Sensograms showing the binding characteristics of 5D3 (A) and 6C3 (B) at different concentrations from 0.2µM to 3.0µM.

mAB	*Ka*	*Kd*	*KA*	*KD*
5D3	1.1e4	1.83e3	6.53e7	1.53e-8
6C3	1.61e4	2.27e3	1.15e6	8.66e-7

Table 3.5: Association (Ka) and dissociation (Kd) rate, and association (KA) and dissociation (KD) constant of anti-MR mAbs (5D3 and 6C3) derived from 5D3 and 6C3 sensogram data using BIA evaluation software 3.1 (Biacore AB) (Myszka and Morton, 1998).

3.3.2 Cloning and sequencing of the variable region of 5D3.

Using as template the total RNA from the 5D3 hybridoma, 5' ready cDNA was synthesised using the method described in Materials & Methods. Both RNA and cDNA were analysed on 1.25% agarose gel to confirm their quality and identity. RNA from the hybridoma showed two bands typically similar to mammalian RNA at 4.5Kb and 1.9Kb corresponding to the 28S and 18S ribosomal RNA (Figure 3.6 A). The region encoding the variable regions of the heavy and light chains of 5D3 were amplified using Taq polymerase with gene specific primers (GSP) (Table 3.2) and universal primers mix (UPM), as described in Materials & Methods. PCR products were separated on a 1%

preparative agarose gel with the V_H of 5D3 appearing as a bright band of ≈750bp, while V_L amplification was suggested by a band of ≈900bp (Figure 3.6 B). Both bands were cut and the DNA was purified.

Gel purified V_H and V_L were cloned into the PCR 2.1 vector (Figure 3.2) and transformed into One Shot competent *E. coli*. The clones were selected on LB-agar plates containing IPTG and X-Gal. 15 white colonies derived from the VL ligation and 25 white colonies derived from the VH ligation were observed. These colonies were further screened by restriction digestion with EcoRI and gel electrophoresis on 1% agarose gel as shown in figure 3.7. Five of the 25 VH clones (VH8, VH17, VH18, VH20 and VH25) and five of the 15 VL clones (VL1, VL6, VL7, VL8, VL11 and VL13) were found positive on the basis of restriction digestion screening. These positively screened clones were sequenced at the Biopolymer Synthesis and Sequencing Facility, University of Nottingham, using of M13 forward and M13 reverse primers.

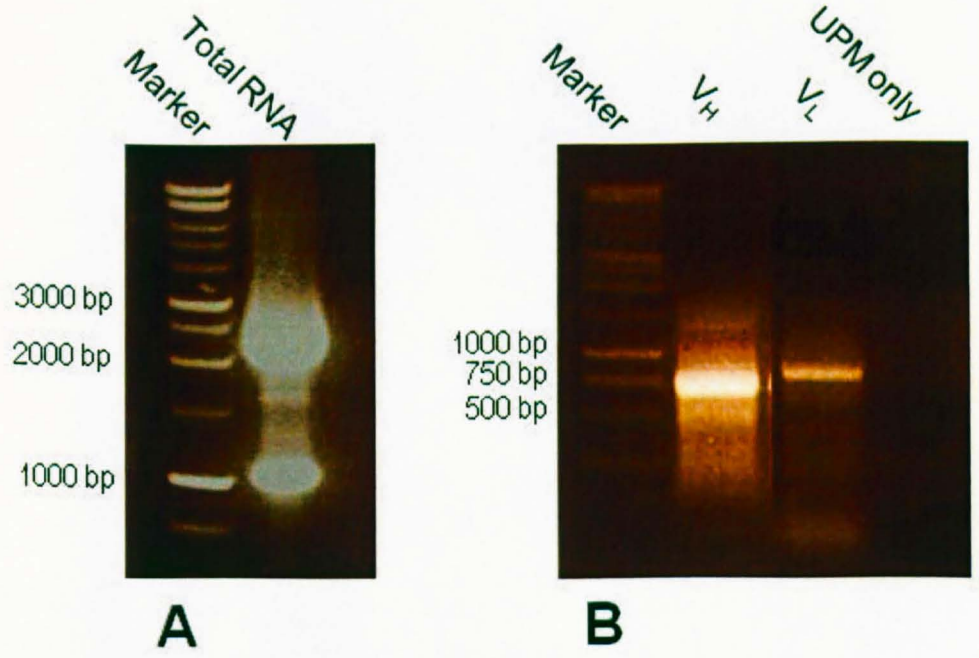


Figure 3.6: Amplification of the variable regions of 5D3 heavy and light chains by 5' Rapid Amplification of cDNA ends (RACE).

(A) Total RNA from 5D3 hybridoma showed two bands typically similar to mammalian RNA at 4.5Kb and 1.9Kb corresponding to the 28S and 18S ribosomal RNA. (B) Products: V_H variable region of heavy chain, V_L variable region of light chain obtained after the final PCR were resolved on 1% agarose gel.

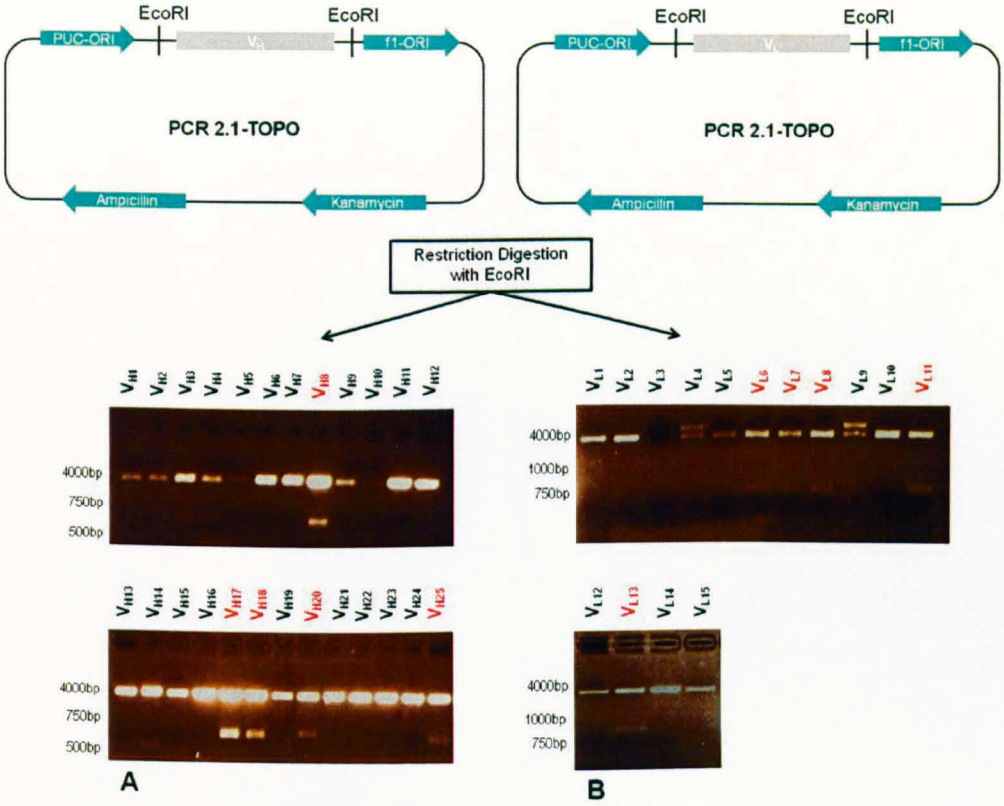


Figure 3.7: Screening of VH and VLclones by restriction digestion.

Plasmids were digested with EcoRI and separated on 1% agarose gel. (A) 25 clones were screened for the presence of heavy chain and (B) 15 clones were screened for the presence of light chain. Products of interest (positive clones) are indicated in red

3.3.3 5D3 hybridoma expresses an aberrant kappa chain

The sequences obtained were analysed using Chromas and SEQ-MAN softwares. All the VH clones showed the same sequences while two distinct types of 5D3 sequences were obtained for clones carrying the light chain variable region. Clones VL7 and VL11 were found similar to the anti-NGF30 antibody kappa chain [Appendix], and were presumed to represent the light

chain of 5D3. Clones VL6, VL8 and VL13 were found to correspond to the myeloma kappa chain found in hybridoma cell line AYG.1.2.3, which could originate from the hybridoma partner (Crowe et al., 1989). The presence of two light chains in the hybridoma expressing 5D3 mAb was confirmed by 2D gel electrophoresis (see section 3.3.3.1).

The variable regions of the heavy and light chains of 5D3 mAb were analysed using the IMGT software (Lefranc et al., 1999) and their nucleotide, amino acid sequence and 2D structures are shown in Figures 3.8 and 3.9. Gene or allele segments used in the variable region of the heavy and light chains of 5D3 are described in Table 3.6.

Antibody	*Isotype*	V_H	*D gene/allele*	J_H	V_k	J_K
5D3	IgG2, k	IGHV8S11*01	IGHD1-8*-01	IGHJ3*01	IGKV11-125*01	IGKJ1*01
DEC205	IgG1, k	IGHV7-3*01	IGHD1-3*01	IGHJ2*01	IGKV15-103*01	IGKJ1*01

Table 3.6: Genes or allele segments used in the heavy and light chains of 5D3 and DEC205 antibodies.

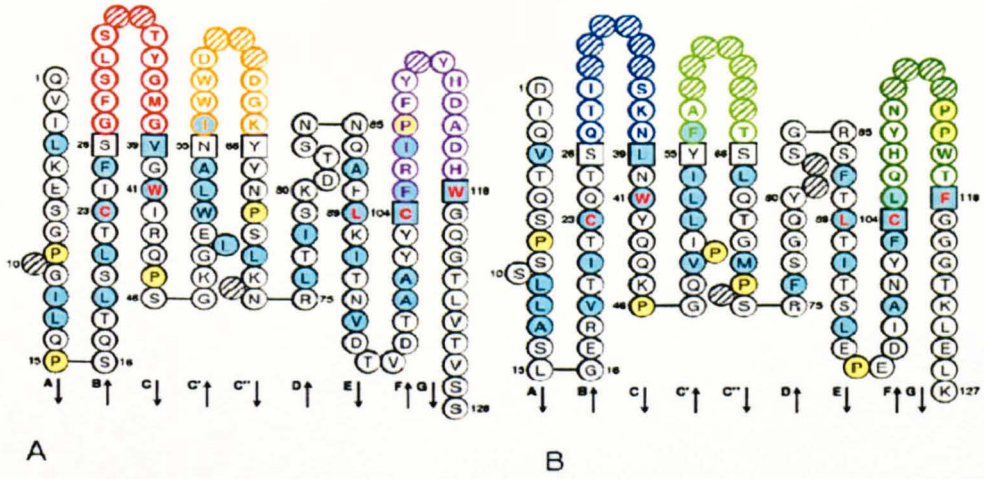


Figure 3.8: 2D graphical representation of the 5D3 heavy chain (A) and light chain (B) generated by the IMGT/Collier de perles tool (Lefranc et al., 1999).

```
5D3H     MDRLTSSFLL LIVPAY-VLS QVTLKESGPG ILQPSQTLSL TCTFSGFSLS TYGMGVGWIR  [ 60]
DEC205H  .G-WSCII.F .VAT.TG.H. E.K.L...G. LV..GGS.R. S.AA...TFN DFY.N--...  [ 60]

5D3H     QPSGKGLEWL ANIWWDDGKY ---YNPSLKN RLTISKDTSN NQAFLKITNV DTVDTAAYYC  [120]
DEC205H  ..P.QAP... GV.RNKGNG. TTEV.T.V.G .F...R.NTQ .ILY.QMNSL RAE...I...  [120]

5D3H     FR-IPFYY-- HDADHWGQGT LVTVSSAETT APSVYPLAPG TALKSNSMVT LGCLVKGYFP  [180]
DEC205H  A.GG.Y..SG D..PY....V M......T.K G......... S.AQT..... ..........  [180]

5D3H     EPVTVTWNSG ALSSGVHTFP AVLQSGLYTL TSSVTVPSST WPSETV---- ----------  [240]
DEC205H  .......... S......... .....D.... S......... ......---- ----------  [240]
```

A.

```
5D3K     MAMKTPAQAL AIWLLWVSGA RCDIQVTQSP SSLLASLGER VTITCQTSQT ISKNLNWYQQ  [ 60]
DEC205K  .GWSC---II LFLVATAT.V HS...M.... .F.ST...NS I....HA..N .KGW.A....  [ 60]

5D3K     KPGQVPILLI YFATSLQTGM PSRFSGQYSG RSFTLTITSL EPEDIANYFC LQHYNPPWTF  [120]
DEC205K  .S.NA.Q... .K.S...S.V ......SG.. TDYIF..SN. Q.....T.Y. QHYQSF....  [120]

5D3K     GGGTKLELKR ADAAPTVSIF PPSMEQLTSG GATVVCFVNN FYPRDISVKW KIDGSEQRDG  [180]
DEC205K  .......... .......... ...S...... ..S....L.. ...K..N... ......RQN.  [180]

5D3K     VLDSVTDQDS KDSTYSMSST LSLTKVEYER HNLYTCEVVH KTSSSPVVKS FNRNEC*---  [240]
DEC205K  ..N.W..... .......... .T...D.... ..S....AT. ...T..I... .......---  [240]
```

B.

Figure 3.9: Protein sequence comparison of the heavy (A) and light chains (B) of the 5D3 and DEC205 antibodies.

Variable regions are shown in blue and the complementary determining regions (CDRs) in the V-region are in green color. Red coloured letters indicate the junction region of the heavy and light chains of the antibodies

3.3.3.1 Identification of the heavy and light chains of 5D3 hybridoma by 2-dimensional Gel electrophoresis

Purified 5D3 was analysed on a reducing SDS-PAGE, and two distinct bands of the mAb heavy and light chains were observed by Coomassie-staining. Amplification and cloning of the variable region of the 5D3 light chain yielded two different sequences, which indicated that the 5D3 hybridoma could contain more than one light chain that might interfere with the cloning of the 5D3 variable region. To investigate this 2D-PAGE analysis of the purified mAbs produced by each of the cell lines was performed (Figure 3.10).

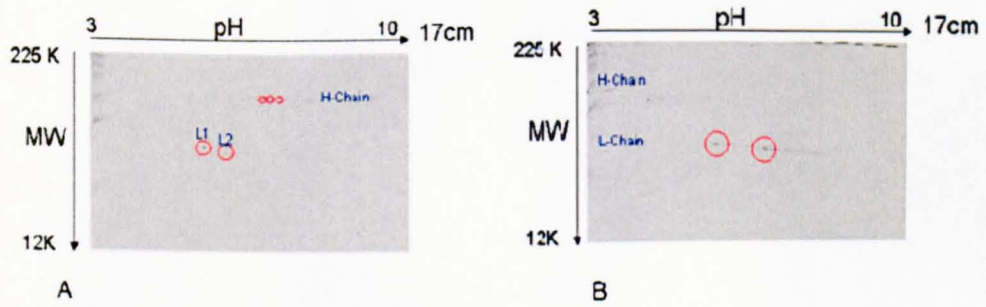


Figure 3.10: Purified mAbs 5D3 (A) and 6C3 (B) were separated on 2D-PAGE in order to identify heavy and light chains being expressed by the hybridoma. Molecular markers are shown on the left side of the gel and the pH range used for the isoelectric focussing is shown at the top.

The light and heavy chains were separated under the reducing conditions of the 2D-PAGE. Multiple spots was observed for the heavy chain, most likely due to the effects of glycosylation and other sources of charge heterogeneity (Perkins et al., 2000). Two spots of different molecular weight, for the light chain were observed coinciding with the results obtained from the Q-TOF sequence analysis (Figure 3.11). In order to confirm whether these two light chains occur by differential glycosylation or if the cell is producing more than one type of light chain the L1 and L2 spots of the 5D3 were analysed by Q-TOF sequencing along with the heavy chain of 5D3.

A.
MAMKTPAQAL AIWLLWVSGA RCDIQVTQSP SSLLASLGER VTITCQTSQT ISKNLNWYQQ [60]

KPGQVPILLI YFATSLQTGM PSRFSGQYSG RSFTLTITSL EPEDIANYFC LQHYNPPWTF [120]

GGGTKLELKR ADAAPTVSIF PPSMEQLTSG GATVVCFVNN FYPRDISVKW KIDGSEQRDG [180]

VLDSVTDQDS KDSTYSMSST LSLTKVEYER HNLYTCEVVH KTSSSPVVKS FNRNEC* [240]

B.
MESQTQVLMS LLLWISGTCG DFVMTQSPSS LAVSAGETVT INCKSSQSLF YSGNQKNYLA [60]

WYQQKPGQSP KLLIYWASTR QSGVPDRFIG SGSGTDFTLT ISSVQAEDLA IYYCLQYYET [120]

PYTFGAGTKL ELKRADAAPT VSIFPPSTEQ LATGGASVVC LMNNFYPRDI SVKWKIDGTE [180]

RRDGVLDSVT DQDSKDSTYS MSSTLSLTKA DYESHNLYTC EVVHKTSSSP VVKSFNRNEC [240]

* [241]
C.
MDRLTSSFLL LIVPAYVLSQ VTLKESGPGI LQPSQTLSLT CTFSGFSLST YGMGVGWIRQ [60]

PSGKGLEWLA NIWWDDGKYY NPSLKNRLTI SKDTSNNQAF LKITNVDTVD TAAYYCFRIP [120]

FYYHDADHWG QGTLVTVSSA ETTAPSVYPL APGTALKSNS MVTLGCLVKG YFPEPVTVTW [180]

NSGALSSGVH TFPAVLQSGL YTLTSSVTVP SSTWPSETV [219]

Figure 3.11: Alignment of the aminoacid sequence of the variable region of 5D3 mAb obtained by 5'RACE and the AA sequence as predicted by Q-TOF analysis.

This diagram shows the protein sequence of the clones carrying the heavy (C) and two light chains (A and B), amplified from the RNA extracted from 5D3 hybridoma using 5'RACE. Peptides identified by Q-TOF analysis of heavy and light chains of the mAb 5D3 are highlighted in grey. Variable region is shown in blue; green text indicates the complementary determining regions within the variable regions; the red and black texts represent the constant regions of the heavy and light chains 5D3 mAb. The text highlighted with grey shade indicates the peptides identified by Q-TOF. (A) Sequence of variable region of 5D3 kappa chain; (C) sequence of 5D3 heavy chain; (B) sequence of hybridoma partner present in the 5D3 hybridoma (Crowe et al., 1989).

3.3.4 Characterization of DEC205-HEL Ab and isotype control IgG1-HEL

DEC205-HEL was used as the backbone to generate the 5D3-HEL chimeric Ab. As the sequence of the vector encoding DEC205-HEL was unknown, it was thus decided to either sequence the complete DEC205-HEL vector, or to clone the sequence encoding DEC205-HEL into another vector with a known nucleotide sequence such as pcDNA 3.1+. Results towards both aims have

been obtained which will be helpful in primer designing and cloning. Endotoxin-free DEC205-HEL and IgG1-HEL plasmids were transfected into the 293T-cells in order to confirm the integrity of the plasmids and the specificity of the expressed antibodies. The original protocol was set up for the transfection of a single plasmid, but two plasmids were required to be transfected at a time. To optimise the transfection the HEK293T-cells were transfected with different DNA concentrations (Figure 3.12). Supernatants were collected and analysed by Western Blotting using anti-mouse IgG-HRP. Consistent expression was observed when using DNA concentrations as in the original protocol, see lanes-A in figure 3.12.

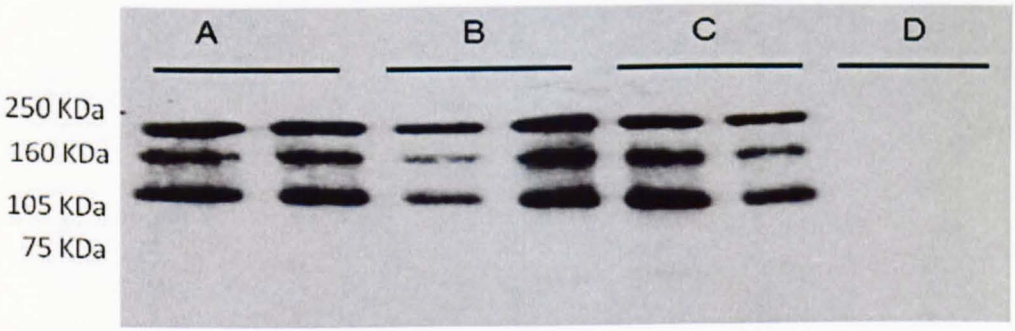


Figure 3.12: Transfection assay to caliberate the amount of DNA in order to attain maximum transfection. Different amounts of DNA from each plasmid were transfected: A (1.00μg), B (1.5 μg), C (2.00 μg), D (negative control).

After the optimisation of the transfection procedure, DEC205-HEL and IgG1-HEL were expressed in HEK 293T-cells and purified from the supernatant by affinity chromatography; 4-5 days post transfection. The concentrations of DEC205-HEL and IgG1HEL were measured by BCA protein quantification assay were found to be 301μg/ml and 91μg/ml respectively. Purified antibodies were analysed by reducing SDS polyacrylamide gel elctrophoresis. Two bands

of molecular weight 52K and 24K were observed which correspond to to the molecular weights of the heavy and light chains of the IgG (Figure 3.13 A).

In order to analyse the specificity of the DEC205-HEL antibody Western Blotting was performed using thymus lysate as it contains a large amount of DEC205 (Witmer-Pack et al., 1995). DEC205-HEL and IgG1-HEL were used as primary antibody and peroxidase-conjugated donkey anti-mouse Ab was used as a secondary antibody. The western blot yielded bands for both IgG1-HEL and DEC205-HEL. IgG1 did not bind the DEC205 receptor appeared to recognised a band that was also recognised by the secondary antibody (peroxidase-conjugated donkey anti-mouse Ab and could correspond to mouse IgG present in the thymus lysate. A band clearly corresponding to DEC205 was observed with DEC205-HEL that was distinct from the band obtained by the binding of secondary antibody (Figure 3.13 C).

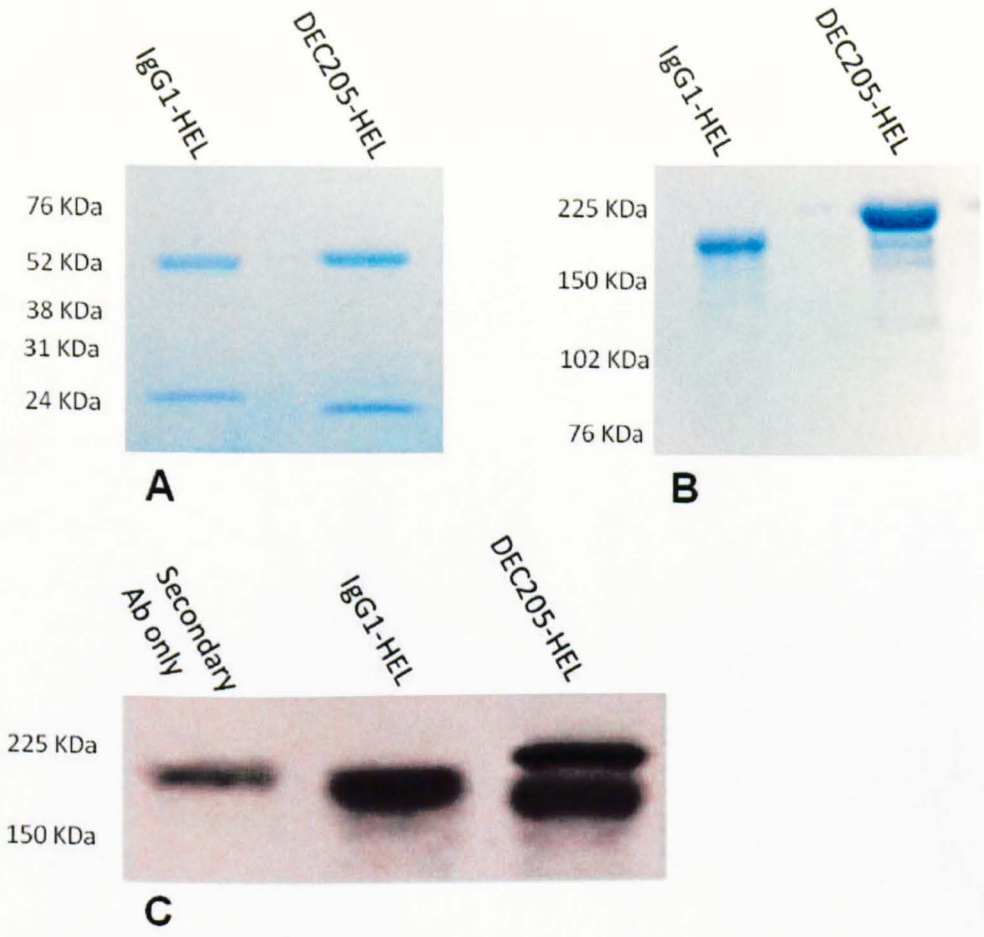


Figure 3.13: SDS-PAGE and Western blot analysis of purified IgG1-HEL and DEC205-HEL.

Antibodies were separated in 12% and 6% acrylamide gels under reducing (A) and non-reducing (B) conditions, respectively, and stained with Simply Blue safe stain. (C) Western Blot of thymus lysates with DEC205-HEL and IgG1-HEL to determine their specificity. Thymus lysate was loaded as sample on 6% SDS-PAGE and DEC205-HEL and IgG1-HEL were used as primary antibodies, and peroxidase-conjugated donkey anti-mouse IgG as secondary antibody.

3.3.5 Construction of the light and heavy chains of the anti-MR chimeric Ab 5D3-HEL

The variable regions of 5D3 were inserted in the place of the variable regions of DEC205-HEL (Appendix1&2) by restriction cloning to generate 5D3-HEL (Appendix3&4). Comparison of the protein sequence of the heavy and light chains of the 5D3 and DEC205 antibodies is shown in Figure 3.16. The variable regions of the 5D3 heavy and light chains were PCR–amplified, with primers containing suitable restriction sites. PCR products were gel purified and digested with EcoRI and HpaI (Light chain) and with EcoRI and ApaI (Heavy chain). DEC205-HEL containing vectors were also digested with the same set of restriction enzymes and ligated into the double digested 5D3 chains (Figure 3.12). Ligation mixtures were used to transform competenT-cells and screened by colony PCR by using product specific primers (Figure 3.14). Almost all of the clones were found positive.

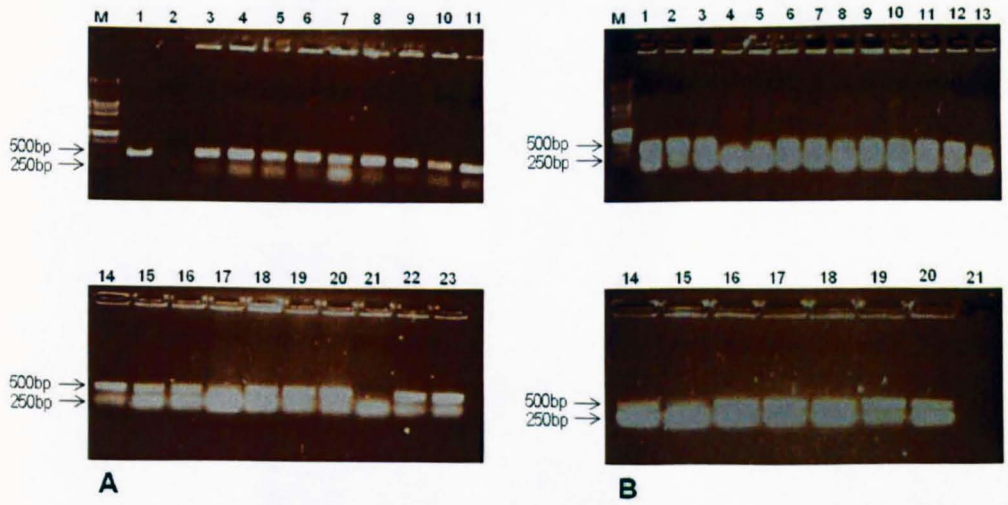


Figure 3.14: Screening of clones carrying the heavy and light chain of 5D3 by colony PCR. PCR-amplified product was separated on 1% Agarose.

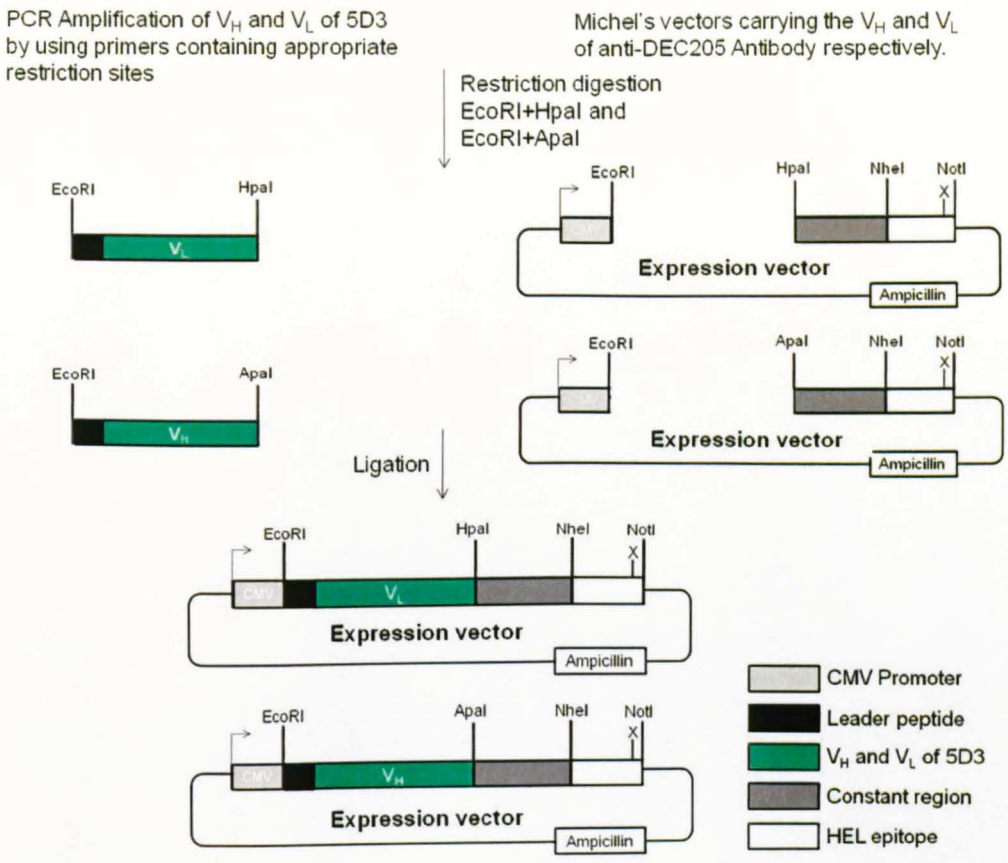


Figure 3.15: Schematic diagram showing the generation chimeric antibody: 5D3-HEL from DEC205-HEL antibody.

3.3.6 The anti-MR chimeric antibody 5D3-HEL binds the mannose receptor

Once the plasmid encoding for 5D3-HEL was generated, it was transfected into HEK293 T-cells for its expression. Antibodies produced were purified from the media by affinity chromatography (Figure 3.16 B). The effect of genetic manipulation on the binding of chimeric antibody 5D3-HEL to CTLD4-7-Fc was assessed using ELISA. Figure 3.16 A demonstrates the binding of 5D3, IgG2a, 5D3-HEL and IgG1-HEL to CTLD4-7-Fc. Isotype control IgG1-HEL showed no binding compared to the anti-MR chimeric Ab 5D3-HEL. Similarly,

the isotype control IgG2a showed negligible binding compared to 5D3 mAb. CR-FNII-CTLD1-3-Fc is used as negative control.

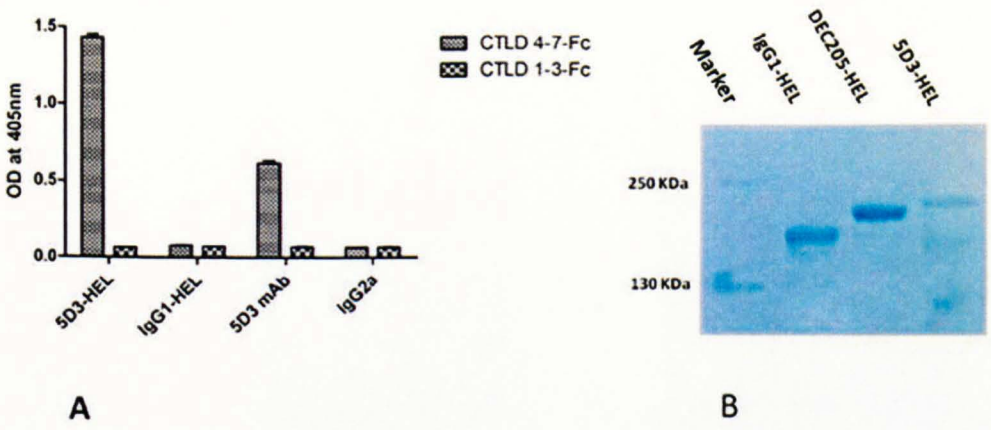


Figure 3.16: SDS-PAGE binding analysis anti-MR chimeric Ab (5D3-HEL) and anti-MR mAb 5D3 with mannose receptor domain CTLD4-7.

(A) The maxisorp ELISA plate was coated with 5μg/ml of CTLD 4-7-Fc and CR-FNII-CTLD 1-3-Fc, followed by the addition of 1μg/ml of primary antibodies. Anti-rat-AP antibody was use as secondary antibody for 5D3 and IgG2a mAb and anti-mouse-AP was used for the chimeric 5D3-HEL and IgG1-HEL. The extent of binding was measured by taking absorbance at 405nm after the addition of 1mg/ml of p-nitrophenyl phosphate substrate. The graph shows OD of the samlples at 405nm and standard deviation (SD). (B) 3μg of the antibodies was loaded on 6% SDS-PAGE and molecular weight (MW) is shown on the left.

3.4 Discussion

Cloning of the VH and VL of the 5D3 mAb is described in this study. An antibody molecule is composed of two (disulfide bond interconnected) heavy chains, each one linked to a light chain by disulfide bonds. It contains a constant region at the C-terminus and an antigen (or complementarity)-determining region (CDR) at the N-terminus, formed by the association of VH and VL. The variable region is futher composed of three hypervariable regions and three frameworks in between them, which are relatively conserved (Ellen Hsu and Lisa A. Steiner, 1992). Cloning of the variable domains of immunogloulins is often carried out by designing 5' degenerate primers: either against frame work-1 (FR-1) sequence or against conservative leader peptide sequences (Gavilondo-Cowley et al., 1990). However, due to the presence of different types of leader peptides and the homology of FR-1 with CDR-3 portions, the use of degenerate primers can result in mispriming and lead to a failure in the cloning of variable regions in 15 to 35% of cases (Doenecke et al., 1997). In order to avoid mispriming the variable region of 5D3 was cloned by designing highly specific primers binding to the highly conserved constant region of rat IgG; the poly-C primer was used against the poly-G tail added at 3' end by 5' RACE PCR in order to obtain a full length V-gene with their native leader peptide. After PCR amplification the VH and VL of 5D3 Ab were cloned into PCR 2.1 vector and sequenced. One of VH was identified on the basis of sequence analysis, with two types of VL clones being identified. 2-D Gel electrophoresis was carried out to detect the presence of different heavy and light chains in the 5D3 mAb molecule; two bands for the light chain were observed similar to the sequencing results wherein two types of VL clones

were identified. Cloning of the V-gene from total RNA isolated from the hybridoma can become complex due to the amplification of irrelevant VH or VK derived from the myeloma fusion partner (Carroll et al., 1988). Q-TOF sequence analysis and DNA sequence blast was performed in order to distinguish between the irrelevant and relevant VL. One VL clone was found to be similar to the hybridoma partner kappa chain (Crowe et al., 1989) and the other VL clone was considered as the VL of 5D3, being further used to generate the chimeric antibody.

The variable region of an antibody (IgG) is assembled by integration of variable (V), diversity (D) and joining (J) segments which is then diversified by somatic hypermuataion (SHM). SHM introduce single nucleotide substituition more frequently in the CDR regions of the IgG to increase the affinity of antibodies for antigen (Di Noia and Neuberger, 2007). VL and VH of 5D3 were analysed by IMGT/V-Quest, a sequence alignment software for IgG (Lefranc et al., 1999), to assess the frequency of SHM in 5D3. In VH of 5D3, V segment indicated 98.63% (287/291 nt) and J segment showed 80.39% (41/51 nt) similarity. While V segement of 5D3-VL indicated 75.27% (210/279 nt) and J-segment had 92.11% (35/38 nt) similarity. The frequency of SHM was more in 5D3-VL as compared to 5D3-VH.

In order to generate 5D3-HEL chimeric antibody, the anti-DEC205-HEL antibody was used as a backbone (Hawiger et al., 2001). DEC205-HEL antibody is a mouse anti-mouse IgG1 and possesses EcoRI and NotI restriction sites at the 5' and 3' ends of both its heavy and light chains. The DNA

sequence of VH and VL of both DEC205-HEL (Appendix-1&2) and 5D3-HEL (Appendix-6&7) was analysed and appropriate restriction sites selected. VL and VH of 5D3 were reamplified by the primers having ApaI for VH and HpaI for VL, which are also present in anti-DEC205-HEL near the variable region. After amplification 5D3 VH and VL were digested with appropriate enzymes and VH and VL of DEC205-HEL were also digested with the same set of enzymes. The variable region of DEC-205-HEL was then replaced with the variable region of 5D3 by DNA ligation (Scharf et al., 1986). The heavy and light chain clones of 5D3-HEL were analysed by colony PCR and DNA sequencing. A stop codon was observed in the heavy chain clones due to a single nucleotide deletion, which was detected as the missing nucleotide in the reverse primer that was used in PCR amplification of VH for restriction cloning (data not shown). This problem was resolved using a new set of primers and clones carrying the heavy and light chains of 5D3-HEL were co-transfected into HEK-293T for protein expression. Unfortunately, we failed to clone variable region of anti-MR mAb, 6C3 by 5' ARCE. However, the techniques learned during the generation of 5D3-HEL chimeric antibody can be employed to mutate the variable region of 5D3 to obtain chimeric antibodies of different binding affinities.

Proteins or antibodies are usually well expressed in bacterial cells as they give good yield (Buchner and Rudolph, 1991). However, such bacterial proteins are not endotoxin-free nor are they properly glycosylated due to the lack of appropriate glycosylation machinery. For this study, therefore, HEK-293T were selected for protein expression. HEK-293T resembles myeloma cells

wherein machinery is maintained intact for antibody synthesis, post-translational modification and secretion. Moreover, proteins expressed in mammalian cells are secreted in the supernatant in a proper folded form (Colcher et al., 1989). The effect of genetic manipulation on the binding of chimeric antibody was assessed by ELISA, and Ag-binding was not affected as reported by Jones et al. that replacement of the variable region of one antibody by one from another does not affect its binding properties (Jones et al., 1986). Although the ELISA result is indicative of conservation of specificity after genetic manipulation, conservation of binding properties will need to be determined using SPR.

4 Investigation of the capacity of chimeric anti-MR Ab to target Ag to antigen presenting cells and induceT-cell immunity

4.1 Introduction

DCs internalize exogenous antigens through different mechanisms such as pincocytosis or receptor-mediated endocytosis, which results in the targeting of soluble Ag to distinct intracellular compartments and antigen presentation to $CD4^+$ T-cells (Burgdorf et al., 2007). Recent studies have shown that the antigen presenting cells, in particular DCs, have the ability to process exogenous antigen and present them on MHC-I molecules. This mechanism is known as cross-presentation and is important for the induction of the immune response against viruses and tumours that are not infecting APC (Heath and Carbone, 2001). DCs located in lymphoid tissues are divided in two subsets: CD8+ and C8- DCs. Among the differences between these cell subsets is that CD8+ DCs preferentially cross-present the exogenous Ag on MHC-I molecules, while CD8- DCs do so on MHC-II molecules (Dudziak et al., 2007).

Following innate stimulation DCs undergo a maturation process that results in the expression of high levels of MHC-peptide complexes and co-stimulatory molecules at the cell surface. Ags loaded on the MHC-peptide complexes are recognised specifically by the T-cell receptor, and co-stimulatory molecules interact with counter-receptors present in the T-cells. These, together with the range of cytokines produced by the DC, influence the outcome of T-cell activation. Therefore, DCs hold the key to the code-controlling the behaviour of the T-cell following interaction (Banchereau and Steinman, 1998).

Once being activated, T-cells perform its cytotoxic role by killing only those tumour or infected cells which display the same antigenic peptide presented by MHC class I molecules (Brodsky and Guagliardi, 1991). Different approaches have been used to target DCs and cross present the exogenous Ags to induce T-cell immunity and antibody mediated targeting of the Ag is the most specific and effective method as described in the general introduction section 1.7.2. Ags can be conjugated to Abs specific to the receptors expressed on the DC by either chemical coupling using cross linkers or by using genetic engineering (as described in Chapter 3).

(A) **Chemical conjugation:** In this method purified Abs are linked with the antigenic peptide by using cross linker molecules such as SMCC (Sancho et al., 2008). This method have been employed in our study as described in chapter number 5 of thesis in which a melanoma Ag, TRP-2 was conjugated chemically to the anti-MR mAbs 5D3 and 6C3 and used to target DCs through MR in order to induce TRP-2 specific T-cell activation.

(B) **Genetic engineering:** Due to possible xenogenic problems DNA encoding the model Ag HEL and OVA Ag have been incorporated into the C-terminus of the constant region of the heavy chain of anti-MR chimeric Ab using genetic engineering (Pudney et al., 2010). DEC205-HEL, 5D3-HEL (Chapter 3) and 5D3-OVA (Chapter 4) along with their isotypes controls have been generated by using this method.

The potential of the novel reagents generated in this study to target DEC205 and MR specifically and to activate T-cells *in vitro* has been investigated. It has

been reported that antigen targeted through MR leads to antigen delivery into EER, a specialised compartment of the cell involved in the cross presentation of the antigens (Burgdorf et al., 2007). Several reports have shown that murine $CD8\alpha^+$ DCs are dedicated to cross prime antigen found in multiple viruses and tumours as well as in tolerogenic immunity (Segura et al., 2009, Villadangos and Schnorrer, 2007). Burgdorf et al showed that $CD8\alpha^+$ DCs express MR and that antigen uptake through MR in BM-DC transported to EER for cross presentation. DEC205 is another receptor expressed by $CD8\alpha^+$ DCs which delivers Ag into EER and leads to cross presentation. But it differs from MR as major part of the antigen delivered through DEC205 was transported to the MHC-II presentation pathway and co-localized with lysosome (Burgdorf et al., 2007).

In vitro Ag presentation assays using HEL and OVA as model antigens were set up in this study. Novel reagents such as 5D3-HEL (chapter 3) and 5D3-OVA (this chapter) have been generated from DEC205-HEL and the potential of these antigen delivery reagents to activate T-cells was assessed, *in vitro* using these assays. DNA plasmids encoding 5D3-OVA and IgG1-OVA have been used to immunise animals to test their potential to generate OVA-specific T-cells.

4.2 Materials and Methods

4.2.1 Replacement of HEL with OVA antigen in 5D3-HEL chimeric antibody to generate 5D3-OVA

pBlueRIP vector carrying the trf-OVA gene was kindly provided by Prof Christian Kurts, Bonn University, Germany. Plamid DNA was recovered and transformed into TOP-10 chemically competent *E.coli*. QIAGEN miniprep kit was used for plasmid isolation aand purified plasmids were characterized by restriction with HindIII. The plasmid included the whole gene of the OVA. A fragment corresponding to a OVA mini-gene was subcloned by using specific primers capable of amplifying a portion of OVA and appropriate restriction sites as shown in table table 4.1. NheI and NotI restriction sites were incorporated into OVA insert using forward primer and revers primers respectively.

The OVA-minigene was PCR amplified using Phusion enzyme (New England Biolabs) and following PCR programme:

95°C 1 min. (Denaturation)
35 cycles: 95°C 30sec, 68°C 1min, 68°C 2min.

Primer name	*Primer sequence*	*GC content (%)*	*Tm (°C)*
OVA-FP (NheI)	TCATAGCTAGCATGTTGGTGCTGTTGCCTGAT	46.9	68.2
OVA-RP (NotI)	TGACAGCGGCCGCTTATGCAGCATCCACTCC AGCC	62.9	75

Table 4.1: Primers designed to subclone OVA mini-gene which include regions complementary to the OVA-minigene and appropriate restriction sites such as NheI and NotI.

PCR products were analysed by agarose gel electrophoresis (1%w/v) and were extracted from the gel by the gel extraction kit Gel extraction kit (QAIGEN) according to manufacturer instructions. This extracted DNA and the 5D3-HEL and IgG1-HEL was digested by using NheI and NotI restriction enzymes according to the manufacturer's instructions. Reactions were incubated at 37°C for 2-4 hours. After restriction digestion the relevant bands were cut and DNA was purified. The digested OVA-minigene was ligated into the 5D3-HEL and IgG1-HEL vector with the help of T4 DNA ligase (Roche). The ligated plasmids were transformed into the One Shot E. *coli* cells. The transformed cells were incubated overnight at 37°C on LB agar paltes containing 100μg/ml ampicilln, 0.1mM IPTG and 40μg/ml X-Gal. White colonies were selected and screened colony by PCR using same set of primer used for the amplification of the OVA-minigene and the following PCR programme:

95°C 15 min. (Cell lysis and denaturation)
35 cycles: 94°C 45 sec, 55°C 45 sec, 72°C 1.5 min.
72°C 10 min. (Final extension)

Positive clones were sequenced at Biopolymer Synthesis and Analysis Unit, Nottingham University using the vector specific primers shown in table 3.4.

4.2.2 Expression of 5D3-OVA and its analysis

Vectors carrying the heavy chain and light chains of 5D3-OVA (Apendix 5&4) and IgG1-OVA (Appendix 7&8) were co-transfected into HEK-293T and the chimeric antibodies were expressed and purified by following the protocol described in section 2.2.1 Purified 5D3-OA and IgG1-OVA were analysed by

SDS-PAGE and western blotting and their binding to mannose receptor was assessed by ELISA as described in section 2.2.4.2.

4.2.3 Characterization of endotoxin free commercial OVA.

4.2.3.1 Lectin ELISA to analyse the binding of commercial OVA to MR

Lectin ELISA and inhibition ELISA were performed as described in sections 2.2.4.2 and 2.2.4.3.

4.2.3.2 Endocytosis assay of commercial OVA

Endotoxin free commercial OVA was labelled with FITC using the Fluoro-Trap flourescein labelling kit (InnovaBiosciences) following manufacturer's instructions. FITC-labelled OVA along with other endocytic tracers (5μg/ml) were used in edocytosis assays following the protocol described in section 2.2.5.

4.2.4 Generation of bone marrow derived dendritic cells.

Bone marrow was collected from C57BL/6 and BALB/C mice according to the protocol described in section and dendritic cells were cultured in R10 medium supplemented with GM-CSF, following protocol described in section 2.3.1. Phenotype of BMDCs was analysed by flowcytometry according to the method described in section 2.2.7.

4.2.5 Maitenance of T-cell hybridoma cell lines

The B3Z hybridoma cell line (Karttunen et al., 1992), a kind gif from Dr. Andrew Jackson, University of Nottingham was maintained in R10 medium.

The suspension cell line was maintained by passaging 1:25 every second day in T75 tissue culture flasks at 37°C in 5% CO_2. The MF2 hybridoma cell line was maintained in R10 medium by passaging every second day while the cells are still subconfluent in T75 tissue culture flasks at at 37°C in 5% CO_2.

The 1C5.1 hybridoma cell line was cultured in R10 medium at 37°C in 5% CO_2. The cell suspension was passaged 1:4 every second day in T75 flasks trying to keep the cells subconfluent.

4.2.6 Antigen presentation assays

4.2.6.1 Antigen presentation assay using B3Z T-cell hybridoma

B3Z cells were harvested while still subconfluent and viable cells counted using Trypan blue (SIGMA) exclusion. Cells were resuspended to a density of $5x10^5$ per ml in R10 media and 100µl were added into the wells of a 96 well flat-bottomed plate (i.e. $5x10^4$ cells per well). Live APC (BMDCs) were resuspended to a density of of $5x10^5$ per ml in R10 media and 100µl were added into the wells of flat-bottomed plate (i.e. $5x10^4$ cells per well) or 10^6 per ml and 50µl were added into each well (giving APC: T-cell ratio 1:1). 50µl of serial 2- fold dilution series (6-dilutions) from a stock solution of endotoxin free ovalbumin (OVA, Profos; Cat: 321000) at 1mg/ml was added per well in quadruplicate, thereby giving a titration of antigen from a top dose of 0.5 mg/ml. The cultures were incubated for 18 hr at 37°C, centrifuged for 5 min at 296 g and 75µl of supernatant were collected and transferred to a fresh 96 well flat-bottomed plate. The plates were stored at 4°C or frozen at -20°C until required for quantification of IL-2.

An alternative procedure for MHC class I-restricted presentation was performed by following a method used by Burgdorf et al, 2006 for investigation of the cross presentation of soluble OVA to naïve T-cells. Live APC (BMDCs) were re-suspended to a density of $5x10^5$ per ml in R10 and plated 100µl into the wells of a 96 well flat-bottomed plate (i.e. $5x10^4$ cells per well). BMDC were incubated with 100µl of media containing 10 µg/ml LPS or 2µg/ml MPLA for 2 hours at 37°C, 5% CO_2. A series of 2- fold dilution (6-dilutions) of OVA (Profos; Cat: 321000) from 2.5mg/ml stock solution or the peptide SIINFEKL (a kind gift from Dr Andrew Jackson) at a final concentration of 200 nM were added into each wells. After 3-4 hours incubation, the cells were washed with PBS, and fixed with 0.008% glutaraldehyde for 3 minutes. The cells were washed again with PBS and co-incubated with $5x10^4$ B3Z T-cell hybridoma in a 96-well flat-bottom plate at 37°C. After 18 hours, the plates were centrifuged for 5 min at 296 g and supernatants were collected, and transferred to a fresh 96 well flat-bottomed plate.

The antigen presentation of different OVA molecules and subsequent B3Z T-cell proliferation was assessed by measuring the amount of murine IL2 secreted by B3Z in the supernatant of co-cultures by following protocol reported in section 2.2.4.4

4.2.6.2 Antigen presentation assay using MF2-T-cell hybridoma

MF2 T-cells were harvested while still sub-confluent and viable cells counted using Trypan blue (SIGMA) exclusion. Cells were resuspended to a density of 10^6 per ml in R10 media and 50µl were added into the wells of a 96 well flat-bottomed plate (i.e. $5x10^4$ cells per well). Similarly, live APCs (BMDCs) were

resuspended to a density of 10^6 per ml in R10 media and 50µl were added into the wells of flat-bottomed plate (i.e. $5x10^4$ cells per well) giving a APC: T-cell ratio 1:1. Stock solution of commercial endotoxin-free OVA (Profos; Hyglos) and purified 5D3-OVA and IgG1-OVA was prepared and 100µl of serial 2-fold dilution from the stock solution was added per well in duplicates, thereby giving a titration of relevant OVA antigen from a top dose of 40µg/ml to 0.078µg/ml. The co-cultures were incubated for 18hr at 37°C, centrifuged for 5 min at 1200rpm and 75µl of supernatant were collected and transferred to a fresh 96 well flat-bottomed plate. The antigen presentation of different OVA molecules and subsequent MF2 T-cell proliferation was assessed by measuring the amount of murine IL2 secreted by MF2 in the supernatant of co-cultures by following protocol reported in section 2.2.4.4.

4.2.6.3 Antigen presentation assay using the IC5.1 T-cell hybridoma

The antigen presentaion assay was performed as described in section 2.3.5.2 except that BMDC were derived from BALB/C mice and pulsed with Hen Egg Lysozyme (HEL)-related antigens from a top dose of 20µg/ml to 0.0390µg/ml. The APCs were then co-cultured with 1C5.1: MHC-II restricted HEL specific T-cell hybridoma as described above.

4.2.7 Dectection of murine IL-2 from B3Z, MF2 and IC5.1 by ELISA

IL-2 produced in the co-culture was assessed by IL-2 ELISA as described in section 2.2.4.4.

4.2.8 DNA immunisation with plasmids encoding 5D3-OVA and IgG1-OVA

Mice were divided into three groups. Each group was immunised with the plasmid DNA encoding 5D3-OVA or IgG1-OVA using gene gun method. One group was immunised with IB-DNA-OVA (Metheringham et al., 2009, Pudney et al., 2010) plasmid using gene gun method as a positive control. Plasmid DNA encoding antibodies carrying the OVA-mini gene was coated on the 1.0 μm gold particles by following the manufacturer's instructions (Biorad, Hemel Hempstead). DNA particles were administered intradermally into the shaved abdomen of the mice using Helios Gene Gun (Biorad) and each mouse was immunised with 1.0μg of DNA per immunisation. After this primary immunisation, secondary and tertiary immunisations were done one week apart as booster doses. One day after the third immunisation, mice were sacrifice and their blood, lymph nodes and spleens were collected in order to analyse the immune response against OVA.

4.2.9 ELISPOT to detect OVA specific T-cells producing IFNγ.

ELISPOT assay was used to determine the frequency of peptide-specific interferon-gamma (IFNγ)-secreting T-cells in murine splenocytes. The Mouse IFNγ ELISPOT kit (Mabtech) was used as follows.

Coating of plates with capture antibody

On day one MAIP multiscreen ELISPOT plates were sterilized with 15 μl of 70% ethanol per well followed by 4 times washing with 200 μl of sterile distilled water per well. The plates were coated with 10 μg/ml anti-IFNγ capture antibody and incubated at 4°C overnight.

Blocking plates

Next day the capture antibody solution was flicked off and plates were washed 4 times with 200 µl of sterile PBS per well. Plates were blocked by adding 100 µl of complete medium to wells and incubation at room temperature for at least 1 hour.

Preparation of effector cells from spleens

Mice were killed by cervical dislocation. After killing the anilmals were sprayed with 70% ethanol and all further procedures were carried out in a laminar flow hood in order to maintain the sterility.

The mouse was laid on its right side, on a paper towel, in the hood. The skin of the left side of the mouse was picked with large forceps and cut with large scissors. The peritoneum cavity was opened and spleen was collected. The spleen was transferred to a petri dish containing 10 ml R10 medium, warmed to 37°C. The outer capsule of the spleen was punctured with 21G needles and the splenocytes were flushed out of the spleen using syringe filled with complete medium. The spleen capsule was gently squeezed with forceps to remove remaining splenocytes. The clumps of splenocytes were allowed to settle for 5 minutes then the cell suspension was decanted into a fresh 25 ml conical universal tube followed by centrifugation at 233 g for 10 minutes in a benchtop centrifuge. The cells pellet was resuspended in 10 ml complete medium containing 2-mercaptoethanol and trypan blue was used to count viable cells using a FastRead disposable haemocytometer. After counting the cells were resuspended at a concentration of 5 x 10 6 cells/ml in complete medium containing 2-mercaptoethanol and $5x10^5$ cells were added per well. SSINFEKL peptide diluted appropriately in complete media plus mercaptoethanol (1 µg/ml

to 0.000001 μg/ml range) were added to ELISPOT plate and incubate at 37°C for 40hrs.

Development of ELISPOT

On day four the development of the ELISPOT plate was carried out on the bench in non-sterile conditions.

ELISPOT plates were washed 5 times with PBS-0.05%Tween. Biotinylated detection antibody (monoclonal antibody R4-6A2 – provided with ELISPOT kit) diluted 1:1000 in PBS was added to the ELISPOT plate and incubated at room temp for 2.5 hrs (2-4hrs).

Next the plates were washed five times with PBS 0.05% Tween followed by the incubation with streptavidin alkaline phosphatase-cojugate (diluted 1/1000 in PBS) for 1.5 hr (1-2 hrs) at room temperature. The plates were washed again six times with PBS 0.05% Tween-20 and was developed by adding 50 μl per well of the development solution and leaving the plate in dark at room temperature for 35 mins (20-45 mins). Once the spots are visible, the plates were washed under tap to stop the reaction. Then, they were dried by keeping the plates at room temperature and the spots were counted.

4.3 Results

4.3.1 Antigen targeting through MR leads to antigen specific $CD4^+$ and $CD8^+$ T-cell activation.

4.3.1.1 Endotoxin free commercial OVA binds MR and shows MR mediated endocytosis.

OVA is commercially available in an endotoxin free form and used as a model antigen in our study. Before we analyse MHC-II specific and cross presentation of OVA through MR in DCs, we assessed first whether OVA bound to MR by lectin ELISA. The plates were coated with either OVA or control sugars of which we used mannan, SO_4-3 Gal-PAA and Gal-PAA. Two different recombinant MR proteins were used with CTLD4-7-Fc preserving the mannose binding properties while CR-FNII-CTLD1-3-Fc was used as negative control. Anti-human IgG Fc-specific, alkaline phosphatase conjugate were used to detect binding. From figure 4.1A, can be observed that OVA bound the MR-construct (CTLD4-7Fc) as mannan did, but not the other MR construct which interacted with the sulphated sugar. As expected none of the proteins recognised galactose.

In order to investigate whether the binding of CTLD4-7-Fc to OVA is mannose-dependent we performed an inhibition lectin ELISA. The MR construct (CTLD4-7Fc) 2μg/ml was incubated with or without competing sugars. Results showed that binding of OVA to CTLD4-7-Fc is mannose dependent, as shown in figure 4.1B.

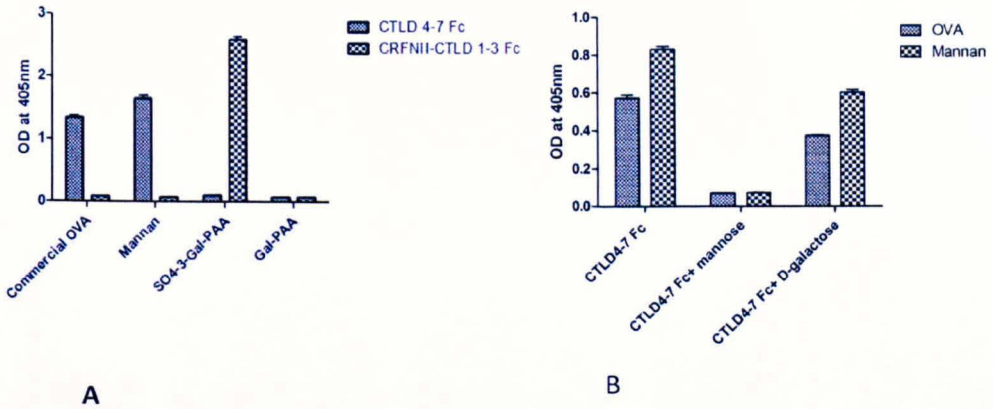


Figure 4.1: Lectin ELISA (A) and Inhibition ELISA (B) showed that OVA is a good ligand for MR.

OVA as well as mannan, a polymer of mannose showed the strongest recognition by the mannose receptor. OVA did not bind to CR-FNII-CTLD1-3Fc as this protein is specific for sulphated sugars galactose was not recognised by CTLD4-7-Fc or CR-FNII-CTLD1-3-Fc. Inhibition Lectin ELISA was used to analyse if the binding of OVA was sugar dependent. The binding of OVA to CTLD4-7 is mannose dependent as CTLD4-7-Fc incubated with 50 mM mannose showed the least binding to OVA (B).

Endocytosis assay was carried out using CHO cells expressing MR. These cells displayed specific uptake of OVA and FITC-labelled Mann-PAA compared to the control CHO cells (Figure 4.2). Thus, we can conclude that OVA is a good ligand for MR and its interaction is mannose dependent.

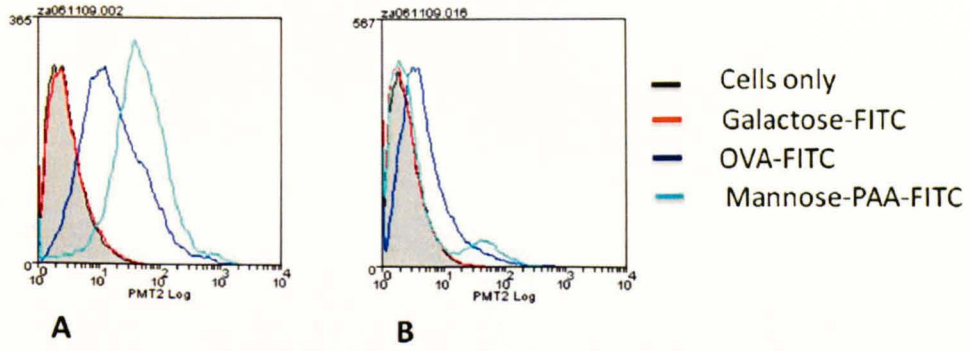


Figure 4.2: Assessment of MR mediated endocytosis by using CHO-MR cells.

CHO cells expressing MR were used to see whether OVA is being endocytosed or not through MR. Mannose-PAA-FITC was used as positive control and represented by light blue histogram while galactose represented by red histogram was used as a negative control. OVA was labelled with FITC by using Fluoro-Trap fluorescein labelling kit (InnovaBiosciences) by following manufacturer's instructions and it is represented by dark blue histogram. Residual uptake by CHO cells was observed indicating the presence of another receptor capable of OVA uptake.

4.3.1.2 Generation of murine BMDCs expressing MR

As mentioned in the methods section, DCs were cultured from the bone marrow obtained from both WT and MR-deficient mice using R10 media supplemented with mouse GM-CSF. DCs were cultured for one week and media was refreshed at day-3 and day-6. After a week, DCs were ready to use and could be seen floating in the form of clusters and loosely attached to macrophages in the TC plates (Figure 4.3A). Moreover, the expression of MR on BMDCs was confirmed by western blot analysis and flowcytometry. Western blot analysis shown in figure 4.3B, implies that MR was expressed by DCs derived from BM of WT C57BL/6 mice but not in the knock out mice.

We examined the phenotype of BMDCs cultured in the presence and absence of LPS by investigating the expression of different markers using flow cytometry analysis. We can see the differences between wild type and knockout mice were only at the extent of MR expression as Burgdorf et al. had

demonstrated that MR is highly expressed in BMDCs grown with GM-CSF (Burgdorf et al., 2006). Interestingly, both populations displayed similar expression of CD11c which is a marker of conventional DCs, indicating that both had the expected phenotype. Moreover, the expression of the maturation markers such as CD80 and CD86 was increased dramatically in the presence of LPS in both wild type and MR-/- BMDCs as shown in figure 4.4.

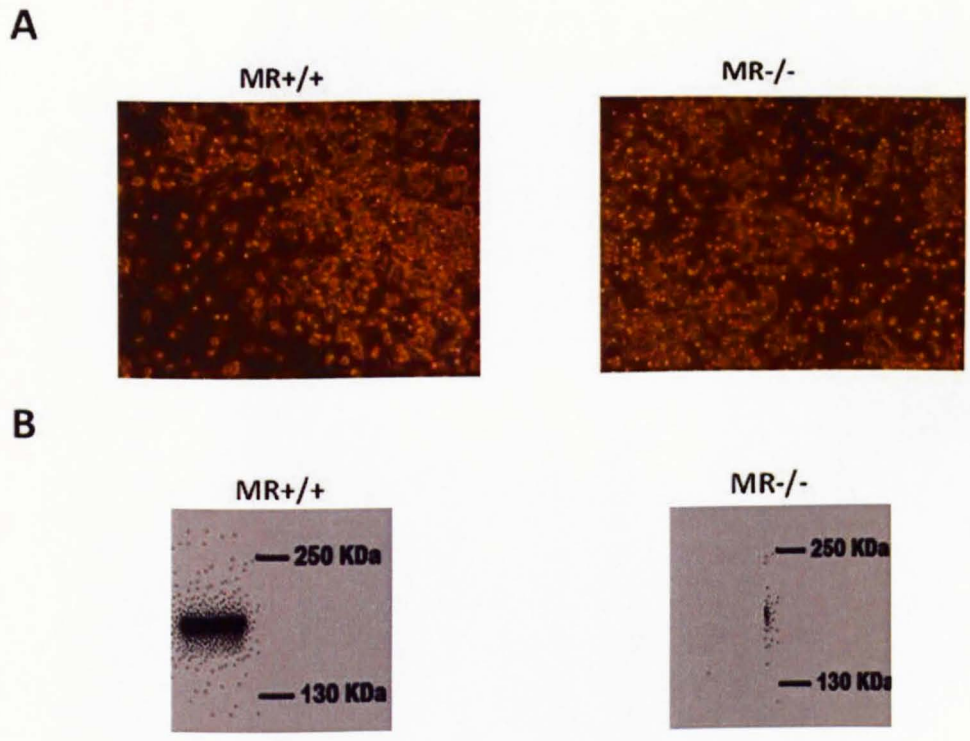


Figure 4.3: Pictures showing the appearance of BMDC cultures from both WTand MR knockout mice (A) and western blot analysis to detect MR (B).

(A) BMDCs were cultured in RPMI-1640 supplemented media with GM-CSF and pictures were taken on day-7. Nice clusters of BMDCs expanding over a layer of firmly adherent macrophages could be observed in both MR+/+ and MR-/- cultures. (B) In order to detect MR expression in BMDCs, 5×10^4 cells of both MR+/+ and MR-/- were lysed and loaded on 6% PAGE. MR was dectected by using 5D3 mAb as a primary antibody and anti-rat IgG labelled peroxidase enzyme as a seconday Ab by using the protocol described in section 2.2.3.

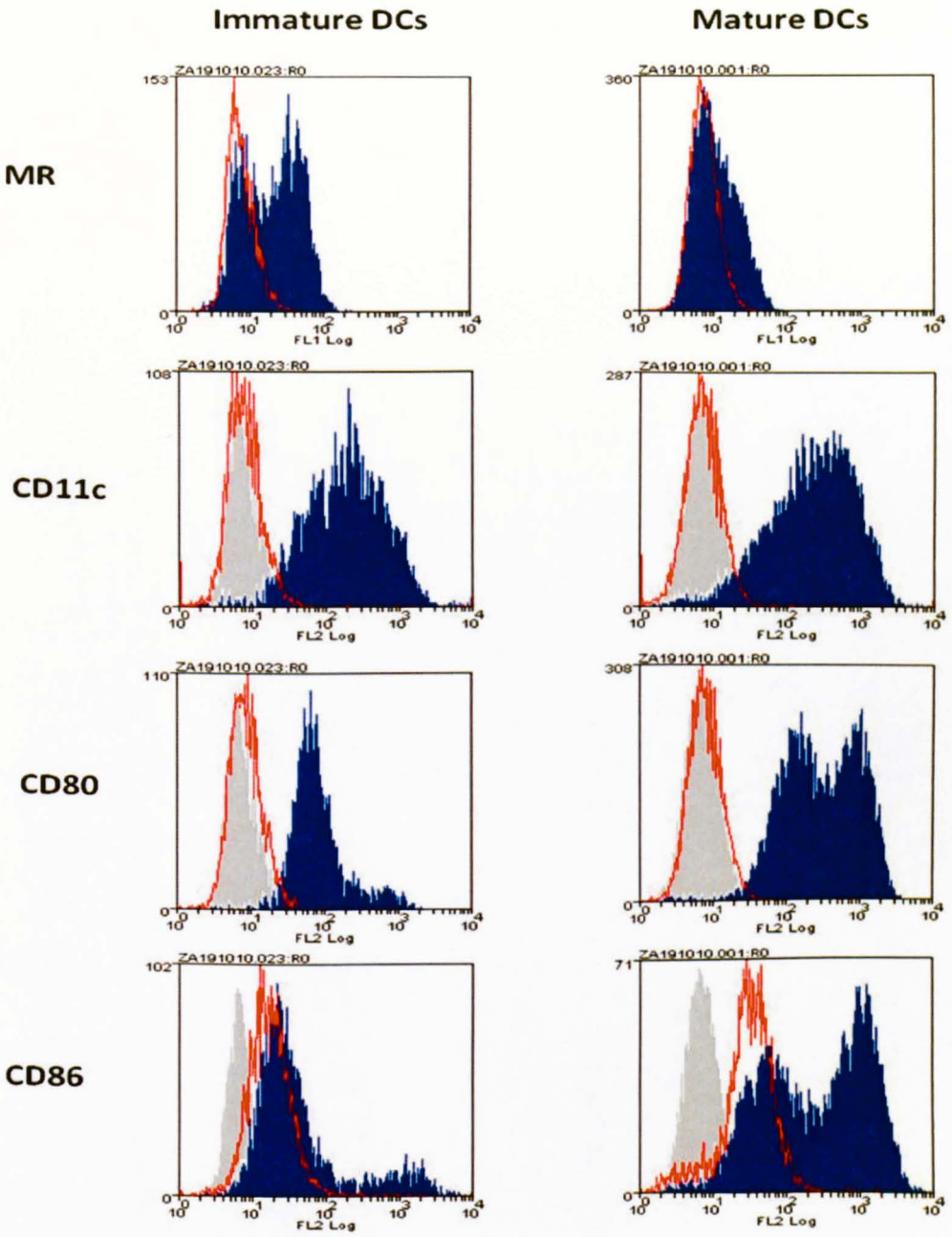


Figure 4.4: Flow cytometry analysis showed the upregulation of different markers on BMDC surface during maturation.

BM derived from CB57/BL6 mice was cultured on RPMI 1640 supplemented with GMCSF and collected on day 7. BMDC were cultured in the presence and absence of LPS (10μg/ml) for four hours. Expression of CD11c, CD80, CD86 and MR was analysed by FACS. In each box, the grey histogram plotted for cells only, the red one represents the background staining with isotype matched antibodies, while the the blue histogram indicates the markers used for staining.

4.3.1.3 Murine BMDCs present OVA to the OVA-specific MHC-I-restricted B3Z T-cell hybridoma

After analysing that murine BMDCs express MR and that the model Ag OVA binds MR, we examined whether Ag targeted to BMDCs through MR leads to Ag prersentation and activation of Tcells. In order to assess T-cell activation an antigen presentaion assay was performed in which BMDCs fed with endotoxin-free OVA were co-cultured with the B3Z cells, OVA-specific $CD8^+$ T-cell hybridoma at a 1:1 ratio. Antigen specific T-cell activation or proliferation was quantified by measuring IL-2 cytokine produced in the supernatants from the co-culture assay. LPS, a TLR-4 agonists was used in this study to aid the maturation of DCs and promote the $CD8^+$ T-cell priming.

In this assay, OVA peptide SIINFEKL was used to confirm that DCs were capable of presenting this peptide to the B3Z cells and that the B3Z cells were able to produce IL-2 in response to the peptide. The peptide used is an octamer derived from OVA and can be presented by the class I MHC molecule, H-2Kb (Carbone and Bevan, 1989). When loaded with SIINFEKL, we can see the BMDCs from MR deficient mice promoted similar activation of the B3Z hybridoma to the one seen with WT cells (Figure 4.5) consistent with results shown in a previous study (Burgdorf et al., 2006). This indicates our BMDCs could still activate the T-cells even without having the MR and from here also we can confirm that our B3Z cell line was working well.

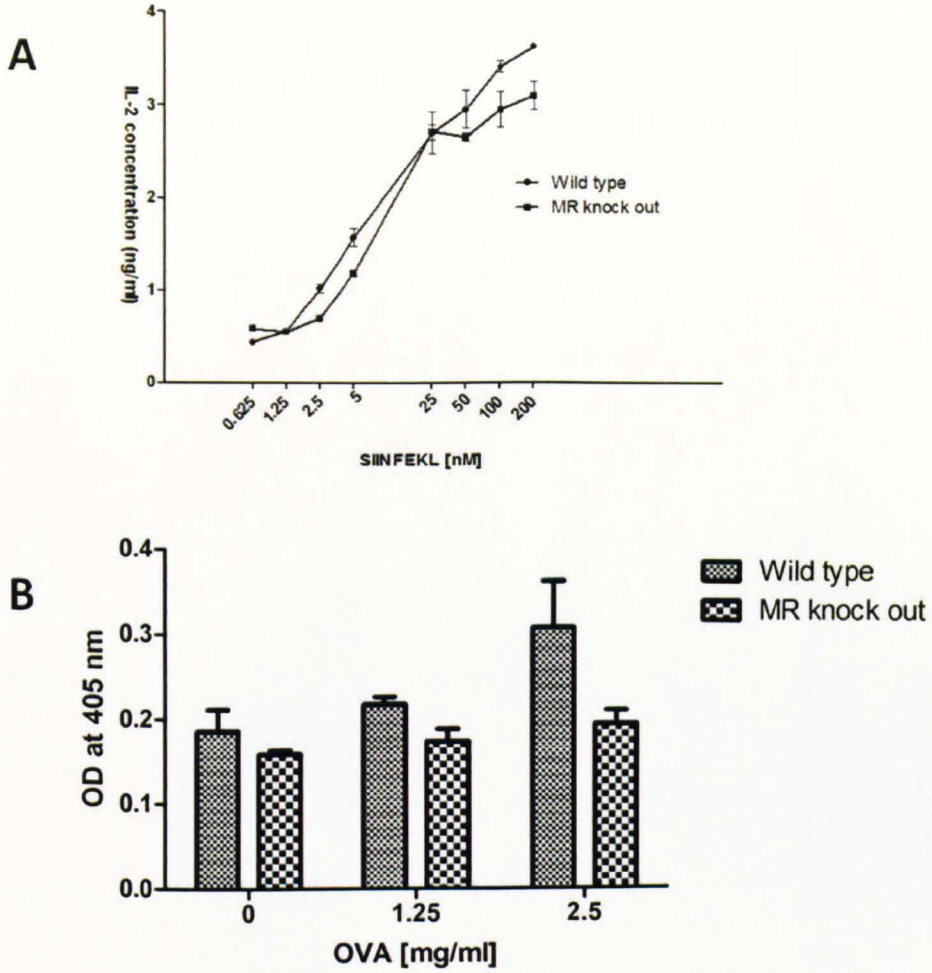


Figure 4.5: (A) SIINFEKL a CD8 epitope of OVA induces T-cell activation measured as IL-2 secretion by the OVA-specific CD8 T-cell hybridoma (B3Z). Graph B shows OD readings which represent different IL-2 cytokine productions induced in response to commercial OVA.

BMDCs from MR+/+ and MR-/- mice were loaded with SIINFEKL at different concentrations from a top dose of 200 nM to 0.625 nM (A) and commercial OVA from 2.5mg/ml to 1.25mg/ml (B). LPS (10μg/ml) was used to induce DC maturation. BMDCs were fixed after 3 hours and co-cultured with B3Z Tcells overnight at 37°C and 5% CO_2. The level of OVA specific T-cell proliferation was estimated by measuring the IL-2 cytokine in the supernatant of the co-culture. The error bar on each column indicates SEM.

Cross presentation assay was performed in which BMDCs were treated with 10μg/ml LPS before adding OVA and OVA peptide SIINFEKL following the procedure described in 4.2.6.1. After 3 hours, DCs were fixed with 0.008% of

glutaraldehyde. Then the B3Z cells were added at a 1:1 ratio and the IL-2 in the supernatants was measured after 18 hours.

Although we found a very poor OVA cross presentation in the assay (Figure 4.5 B), it is quite interesting to see all the readings from MR deficient BMDCs seemed to have an identical B3Z activation (OD=0.15), but increasing levels of IL-2 production with increasing dosage of OVA was observed when using WT-cells. This might indicate that MR-dependent antigen cross- presentation by BMDCs was taking place although MR-/- also induced less IL-2 production when using the peptide (Figure 4.5 A).

Therefore, from what we have seen in the cross presentation assay, we could not get the B3Z hybridoma activated sufficiently when BMDCs were incubated with OVA and even in the presnce of a TLR-4 agonist.

4.3.2 Generation of anti-MR chimeric antibody carrying the model antigen OVA (5D3-OVA)

The HEL-derived peptide from 5D3-HEL and IgG1-HEL was replaced with a OVA minigene using the primers and strategy described in section 4.2.1 to generate 5D3-OVA and IgG1-OVA. Both proteins were expressed in HEK 293T-cells and after 3-4 days the supernatants were collected and purified by affinity chromatography. The concentration of 5D3-OVA and IgG1-OVA was measured by BCA protein quantification assay. Purified antibodies were analysed by reducing and non-reducing SDS-PAGE. Two bands of molecular weight 52K and 24K were observed which are similar to molecular the weight of heavy and light chain of the IgG (Figure 4.6C).

In order to confirm the replacement of HEL with OVA western blotting was performed using anti-OVA polyclonal Ab. Both 5D3-OVA and IgG1-OVA were detected by this Ab which indicated that these chimeric antibodies included the OVA antigen. As expected, both chimeric antibodies were also detected by a peroxidase-conjugated donkey anti-mouse Ab as 5D3 is a rat mAb while the chimeric Ab is mouse Ab and a band observed indicated these chimeric Abs are of mouse origin (Figure 4.6B).

The effect of genetic manipulation on the binding of 5D3-OVA to CTLD4-7-Fc was assessed by using ELISA. Figure 4.6A demonstrates the binding of 5D3, IgG2a, 5D3-OVA and IgG1-OVA to CTLD4-7-Fc. Isotype control IgG1-OVA showed no binding compared to the anti-MR chimeric Ab 5D3-OVA. Similarly, isotype control IgG2a showed very negligible binding as compared to 5D3 mAb.

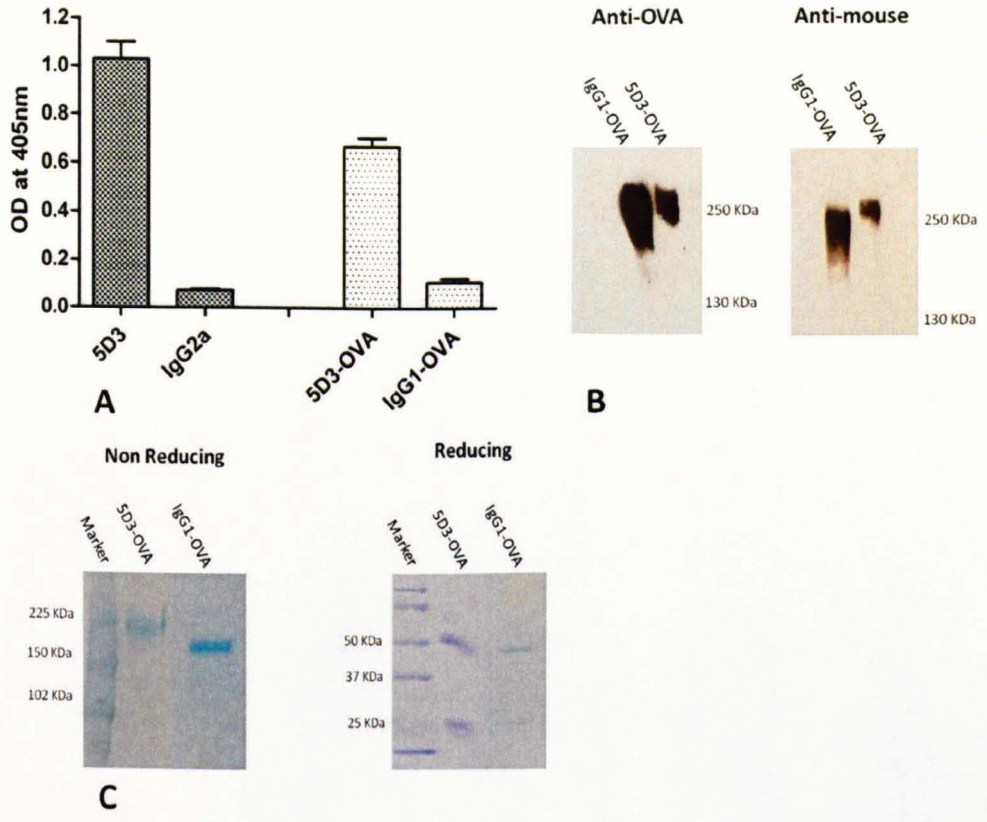


Figure 4.6: Binding properties, SDS-PAGE and Western blot analysis of purified chimeric antibodies IgG1-OVA and 5D3-OVA.

(A) The maxisorp ELISA plate was coated with 5μg/ml of CTLD 4-7Fc and CR-FNII-CTLD 1-3Fc followed by the addition of 1μg/ml of chimeric anti-MR antibodies. Anti-rat-AP antibody was use as secondary antibody for 5D3 mAb and anti-mouse-AP was used for the chimeric 5D3-OVA. The extent of binding was measured by taking absorbance at 405nm after the addition of 1mg/ml of p-nitrophenyl phosphate substrate. The graph shows OD of the samlples at 405nm and standard deviation (SD). (B) Western blot analysis in which chimeric Abs were loaded as sample on 6% acrylamide gel and later detected by using anti-OVA and anti-mouse IgG. (C) Chimeric Abs were separated in 12% and 6% acrylamide gels under reducing and non reducing conditions respectively and stained with Simply Blue safe stain.

4.3.3 Chimeric antibody 5D3-OVA induce activation of the MF2 T-cell hybridoma

Commercial OVA is mannosylated and shows binding to MR: due to these reasons it has been used as model antigen in vaccination studies involving MR-mediated Ag targeting and the activation immunity. Due to broader binding

range of mannosylated Ags, anti-MR Ab carrying the OVA minigene (5D3-OVA) has been generated for specific delivery of Ag through MR in order tod assess its potential to promote T-cell activation. *In vitro* antigen presentation assay (n=1) was performed to assess the ability of 5D3-OVA to activate MF2 hybridoma a MHC-II-restricted OVA specific T-cell hybridoma, by co-culturing of BMDCs and MF2 in the presence of 5D3-OVA and IgG1-OVA as control. Murine BMDCs were mixed with MF2 T-cell hybridoma at a 1:1 ratio and cultured in the presence of commercial OVA, 5D3-OVA and IgG1-OVA titrated using two fold dilutions from a top dose of 40μg/ml to 0.0781μg/ml. MF2 T-cells and DCs alone were considered as negative controls and the plates were incubated at 37°C with 5% CO_2. Supernatants were collected after 18 hours of incubation and the amount of IL-2 produced was assessed by ELISA. IL-2 is a T- cell proliferation marker and negative controls such BMDCs only, MF2 cells only did not show any IL-2 production. No IL-2 production was observed in the absence of antigen while in the presence of commercial OVA, 5D3-OVA and IgG1-OVA BMDCs induced IL-2 production in a dose dependent manner as shown in figure 4.7.

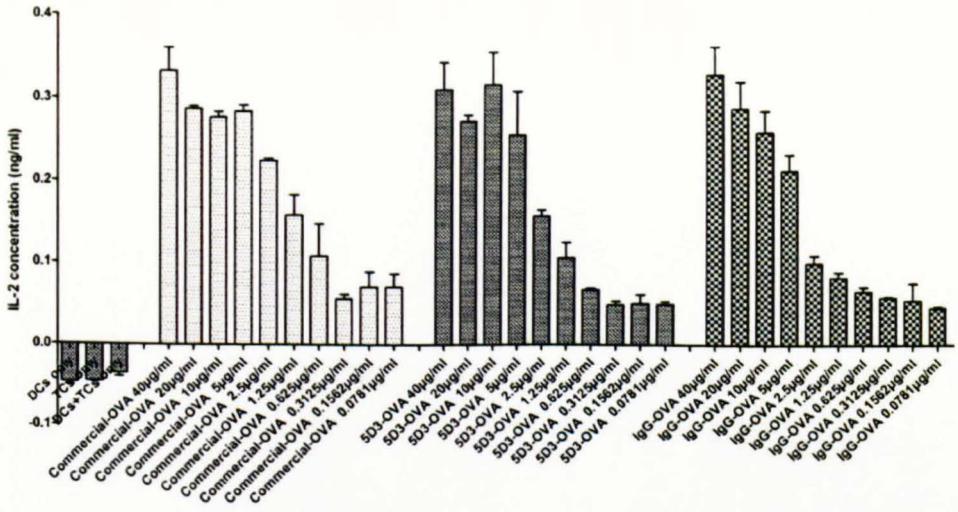


Figure 4.7: T-cell proliferation assay by co-cultures of BMDC and MF2: OVA specific $CD4^+$ T-cell hybridoma in response to commercial OVA, 5D3-OVA and IgG1-OVA.

BMDCs obtained from C57BL/6 mice were fed with 5D3-OVA, IgG1-OVA and commercial OVA at different concentrations from top dose of 40µg/ml to 0.0781µg/ml and co-cultured with MF2 Tcells overnight at 37°C and 5% CO_2. The level of OVA specific T-cell proliferation was estimated by measuring the IL-2 cytokine in the supernatant of the co-culture. The error bar on each column indicates SEM (n=2).

Surprisingly T-cells produced the same IL-2 levels when incubated with DCs in the presence of IgG1-OVA and 5D3-OVA indicating that MR binding by 5D3-OVA does not lead to enhanced T-cell activation which was also similar to that obtained with OVA. To confirm that the IgG1 portion of IgG1-OVA had not been modified during the cloning process, we tested the Ag presentation capability of the proteins encoded by the original plasmids supplied by the laboratory of Prof. Michel Nussenzweig.

4.3.4 Activation of the HEL-specific 1C5.1 T-cell hybridoma in the presence of DEC205-HEL and IgG1-HEL

Similarly as with OVA-derived antigens, antigen presentation assay has been performed with HEL. *In vitro* antigen presentation assay was performed to assess the ability of DEC-HEL, 5D3-HEL (chapter 3) and IgG1-HEL to activate the MHC-II restricted HEL specific T-cell hybridoma 1C5.1. Balb/c BMDCs were mixed with 1C5.1 T-cell hybridoma at a 1:1 ratio and cultured in the presence of low endotoxin HEL, DEC205-HEL, 5D3-HEL and IgG1-HEL titrated using two fold dilutions from a top dose of 20μg/ml to 0.039μg/ml, at 37°C with 5% CO_2. 1C5.1 T-cells alone, DCs alone and DCs with T-cells in the absence of antigen were cultured as the negative controls. Supernatants were collected after 18 hours of incubation and the amount of IL-2 produced was assessed by ELISA. IL-2 is a T- cell proliferation marker and negative controls such BMDCs only, MF2 cells only did not show any IL-2 production. BMDCs did not induce any T-cell proliferation i.e IL-2 production in the absence of antigen while in the presence of HEL, 5D3-HEL and IgG1-HEL BMDCs induced IL-2 production in dose dependent manner as shown in figure 4.8.

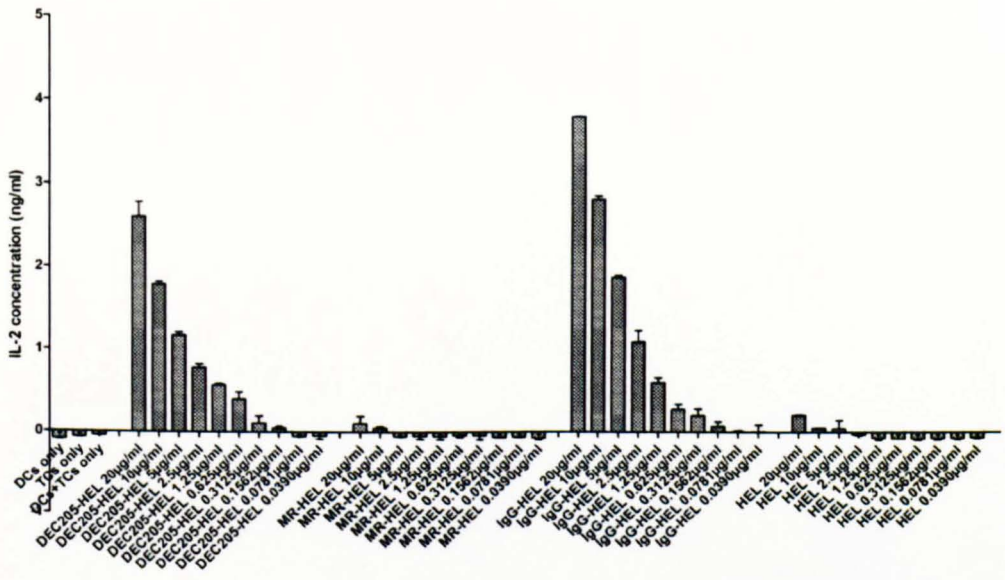


Figure 4.8: Contribution of antibody-mediated targeting of the model Ag HEL to T-cell proliferation assay.

DCs ($5x10^4$ cells) obtained from the bone marrows of BALB/C mice were fed with DEC205-HEL, 5D3-HEL (MR-HEL), IgG1-HEL and low endotoxin HEL at different concentrations from top dose of 40μg/ml to 0.0781μg/ml and co-cultured with $5x10^4$ 1C5.1 T-cells overnight at 37°C and 5% CO_2. The level of HEL specific T-cell proliferation was estimated by measuring the IL-2 cytokine in the supernatant of the co-culture. The error bar on each column indicates SEM (n=1).

These results show that when using BMDC, the IgG1-HEL control was capable of promoting T-cell proliferation to levels similar to those obtained with DEC205-HEL. MR-HEL induced very low T-cell activation most likely caused by the low levels of MR expression we observed in these BMDC cultures (data not shown).

4.3.5 *In vivo* immunisation of plasmid constructs encoding 5D3-OVA by using gene gun method and quantification of OVA-specific $CD8^+$ T-cells

C57BL/6 mice were immunised with DNA plasmids encoding heavy and light chain of 5D3-OVA and IgG1-OVA by Dr. Ian Spendlove. Moreover DNA plasmid encoding human IgG1 in which a CD8 epitope of OVA (SIINFEKL) was incorporated into CDRH2 region was also used for immunisation and used as positive control provided by my co-supervisior, Dr. Lindy Durrant (School of Molecular Medical Sciences, Nottignham University). Splenocytes from the immunised mice were collected and cultured in the presence of SIINFEKL titrated using ten fold dilutions. IFN-γ secreting SIINFEKL specific $CD8^+$ T-cells were counted by ELISPOT. Mice immunized with 5D3-OVA showed higher frequency of activated OVA specific T-cells (Figure 4.9B) compared to mice immunised with plasmid DNA encoding human IgG1 containing a CD8 epitope of OVA (Positive control). Mice immunised with IgG1-OVA (Figure 4.9C) showed highest frequency of IFN-γ secreting OVA specific $CD8^+$ T-cells even more than the positive control and 5D3-OVA (Figure 4.9B). When the splenocytes were cultured in the absence of SIINFEKL peptide, no activated T-cells were observed which indicated that the T-cell activation was OVA specific (Figure 4.9D).

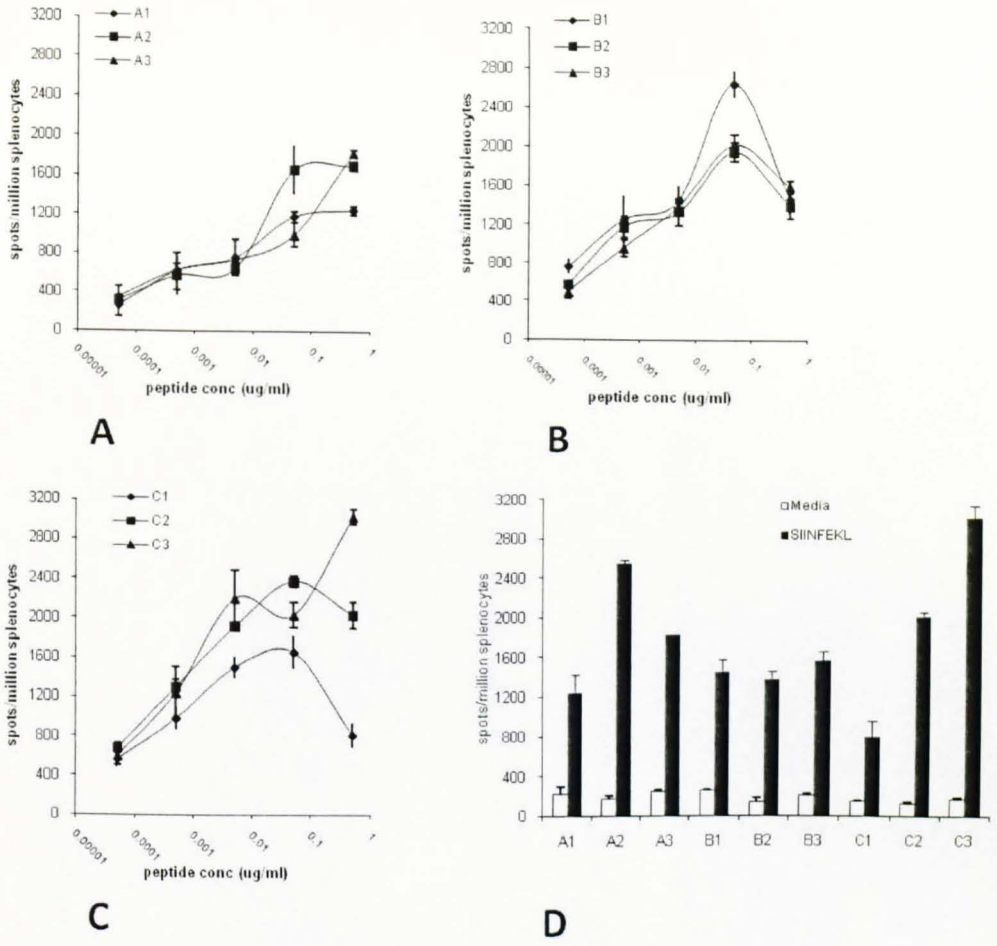


Figure 4.9: Immunisation of C57Bl/6 mice with DNA encoding the 5D3-OVA and IgG1-OVA and the assessment of T-cell activation.

After three vaccinations, splenocytes were collected from the different groups of mice immunised with plasmid DNA encoding 5D3-OVA, IgG1-OVA and human IgG1 containing SIINFEKL in its CDRH2 region. Splenocytes were cultured in the presence of different concentrations of SIINFEKL peptide which is a CD8 epitope of the model Ag OVA. The number of IFN-γ secreting OVA specific T-cells were counted from the culture by using ELISPOT assay in which each spot indicated the IFN-γ secreting OVA specific T-cell. (A) mice immunised with plasmid DNA encoding human IgG1 containing SIINFEKL in its CDRH2 region (positive control) by using gene gun method, (B) mice immunised with plasmid DNA encoding 5D3-OVA, (C) mice immunised with IgG1-OVA and (D) Splenocytes cultured in the presence and absence of SIINFEKL. Graph shows mean spots/million splenocytes plus standard deviation (SD).

4.4 Discussion

4.4.1 Generation of MR specific chimeric Abs, their expression and purification

Anti-MR chimeric antibodies were engineered by using an anti-DEC205 chimeric Ab as starting material. Anti-DEC-205 Ab consists of the variable region of rat anti-DEC-205 variable region and a mutated form of the constant region of mouse IgG1 that reduces binding to FcγRIIB and FcγRIII (Clynes et al., 2000). In order to generate anti-MR chimeric Abs the variable region of anti-DEC-205 Ab was replaced with the variable region of anti-MR mAb thereby minimizing the possibility of these anti-MR chimeric Abs to engage FcγRs. After genetic manipulation, MR specific chimeric Abs retained their ability to bind MR as assessed by ELISA. MR specific chimeric Abs were expressed using HEK-293T however we consistently obtained low level of expression. To circumvent this issue chimeric Abs were expressed at a larger scale. Purified chimeric Abs were analysed by SDS-PAGE and the preparations of chimeric Abs such as 5D3-OVA and 5D3-HEL contained a 140 KDa product that could correspond to a degradation product (indicated by the red arrows in figure 4.10). The protein expression protocol was revised in order to reduce the degradation of chimeric Abs. Firstly, the time of expression was reduced from 4-5 days to 3 days to reduce the cell death and release of cellular proteolytic enzymes. Secondly, protesase inhibitors were used during storage and lastly the whole protein purification was performed in cold room at 4°C. After these series of optimization steps we were successful in geting undegraded MR-specific chimeric Abs as shown in figure 4.6 but still these

Abs were found highly prone to degradation when exposed to room temperature as discussed in chapter 7. Moreover, mouse IgG is difficult to purify as it has poor ability to interact with protein G or protein A compared to human and rabbit IgG. The exchange of threonine 256 residue in the mouse IgG Fc region to methionine can enhance the binding of mouse IgG to protein A to facilitate the efficient purification of mouse IgG, so the purification of our chimeric Abs (IgG1) could be improved by these modifications in their Fc region (Nagaoka and Akaike, 2003).

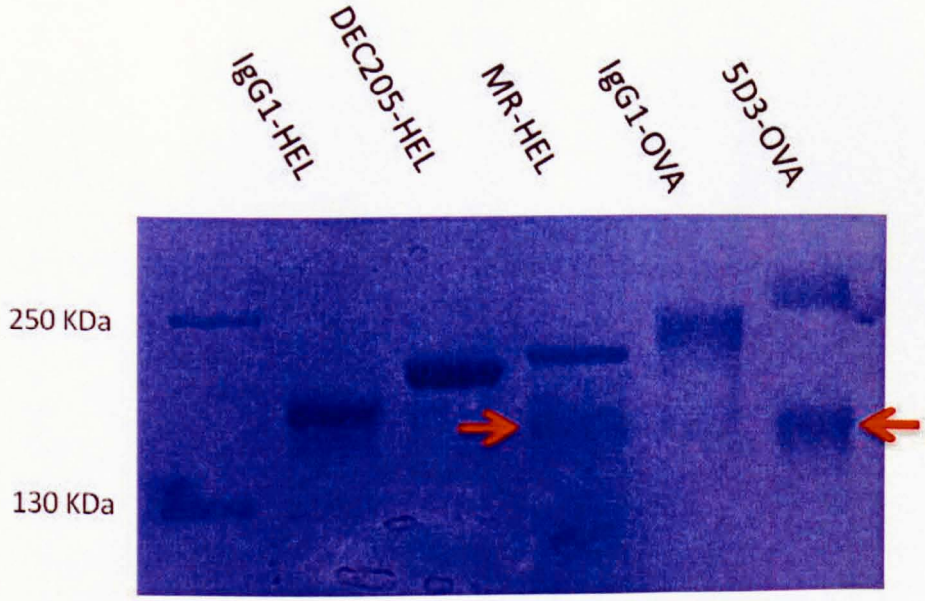


Figure 4.10: SDS-PAGE to test the integrity of the panel of antibodies used in this project. 3μg of antibodies were separated in 6% acrylamide gels and stained with Simply Blue safe stain. Red arrows indicate the position of degraded product of 5D3-OVA and 5D3-HEL Abs. Molecular weight (MW) markers are shown on left.

Once the chimeric Abs were purified, their cellular binding and uptake should have been investigated by using stable transductants expressing full length MR such as CHO-MR cells (Martinez-Pomares et al., 2003) or using DCs and macrophages derived from the bone marrows of WT and MR-/- mice. Moreover, we could have injected MR-specific chimeric Abs into the forelimbs

of mice in order to analyse the potential of these Abs to target MR^+ DCs in the secondary lymphoid organs. The presence of the chimeric antibodies could have been be detected in peripheral lymph nodes by immunohistochemical analysis as described by Mckenzie et al. (McKenzie et al., 2007). But due to the lack of time (the optimization of Abs expression and elimination of the degradation product took a long time) we focussed on antigen presentaion assays to investigate the capacity of chimeric Abs to deliver Ag to APCs and promote T-cells proliferation using T-cells hybridoma such as MF2, B3Z and 1C5.1.

4.4.2 Cross presentation of commercial OVA and proliferation of B3Z T-cell hybridoma.

Functional studies were initiated by testing the ability of BMDC to promote $CD8^+$ T-cell activation when fed with a commercial preparation of OVA using *in vitro* antigen presentation assays with the OVA-specific MHC-I restricted T-cell hybridoma, B3Z. We also demonstrated that OVA acts as a MR ligand (Figure 4.1). Burgdorf et al. (2006) demonstrated that MR is required for OVA cross presentation but they did not show that OVA bound MR. These authors also showed that in both *in vivo* and *in vitro* systems, soluble OVA requires MR for its uptake by DCs and cross presentation (Burgdorf et al., 2006). Our endocytosis assay using CHO cells expressing full length MR, confirmed that the internalisation of OVA by BMDCs (*in vitro*) is likely mediated by MR (Figure 4.2). Thus, we can conclude that OVA binds to MR and its uptake is MR-dependent. BMDCs were generated in our lab by using both wild type and MR deficient knockout mice. Consistent with the earlier study (Burgdorf et al.,

2006) MR was highly expressed by BMDCs generated with GM-CSF (Figure 4.4).

Burgdorf, Scholz et al. (2008) proposed a cross presentation pathway/ mechanism in which MR mediated uptake of soluble model Ag such as OVA by DCs leads to its accumulation in the stable early endosome compartment and facilitates cross-presentation. They demonstrated that endotoxin a common contaminant in OVA preparations is very important to recruit transporter associated with antigen processing (TAP) in the early endosomes. TAP transports Ag to the cytoplasm for its proteolytic digestion into peptides and reimports peptides into the same early endosome compartment for cross presentation (Burgdorf et al., 2008). However, Segura et al. (2010) repoted that MR mediated endocytosis of soluble Ag by DCs leads to cross presentation only in inflammatory conditions but not in steady-state conditions (Segura et al., 2009).

The ability of OVA to be cross-presented was analysed by co-culturing BMDCs and B3Z and measuring IL-2 produced in the supernantant. When MR+/+ BMDC were fed with SIINFEKL peptide (MHC-I epitope) induced the same T-cell proliferation as MR-deficient BMDCs which indicated that the general ability of BMDCs to activate B3Z T-cell hyridoma is not affected by the absence of MR. In our cross-presentation model, very poor activation of B3Z T-cell hybridoma was observed compared to previous studies (Burgdorf et al., 2006, Burgdorf et al., 2007) but still, a minor effect of MR could be observed as shown in figure 4.5.

Several possible reasons could be suggested for the lack of IL-2 productions in our cross presentation model. First, we know that the uptake of soluble OVA is likely mediated by the MR. We could test if in our BMDCs, OVA is also internalised and if this internalisation is MR-dependent. Second, the lack of antigen processing ability of DCs might be the cause of the failure of cross presentation. In order to address this question, BMDCs were co-cultured with MF2 (OVA-specific $CD4^+$ T-cell hybridoma) in the presence of commercial OVA, to investigate whether OVA is being internalised, processed through the MHC class II pathway by the BMDCs used in our co-culture assays. IL2 production by MF2 indicated that BMDC were capable of OVA uptake and processing. Another cause might be the lack of sensitivity of the B3Z T-cell hybridoma. So, the possibility of using naive T-cells isolated from transgenic OT-I cells instead of the B3Z cells might be considered, as it had been shown to be very sensitive to cross-present OVA *in vitro* (Burgdorf et al., 2006).

4.4.3 *In vitro* presentation of OVA and HEL containing reagents to $CD4^+$ T-cells, MF2 and 1C5.1 T-cells hybridomas

Antibodies are more sophisticated and specific tool to target antigen to APCs (Keler et al., 2007). Anti-MR chimeric Ab carrying the model Ag HEL (5D3-HEL) was generated to deliver Ag specifically through MR as glycosylated proteins such as OVA could bind more than one receptor which leads to Ag delivery through several receptors and potentially, a different immune outcome. HEL Ag was replaced with the OVA-minigene in order to generate the chimeric antibodies, 5D3-OVA and IgG1-OVA due to the following reasons. Firstly, OVA has been widely used in MR-mediated Ag presentation

studies, so it would more appropriate to use OVA to compare results obtained in this study with earlier findings in which OVA has been used as a model Ag. Secondly, we had access to OVA specific $CD4^+$ and $CD8^+$ T-cell hybridoma in order to perform Ag presentation studies *in vitro.* Moreover, cancer cells expressing OVA Ag are available to assess the potential of these novel reagents to treat cancer.

The response of the OVA-specific $CD4^+$ T-cells (MF2) was examined in the presence of commercial OVA, 5D3-OVA and IgG1-OVA by using co-cultures of BMDC and MF2. In this assay, as described in results section figure 4.7, we used different doses of commercial OVA and chimeric antibodies carrying OVA but lacked the MR-/- control. Commercial OVA induced antigen specific $CD4^+$ T-cells in a dose-dependent manner similar to 5D3-OVA and IgG1-OVA. $CD4^+$ T-cell proliferation was not enhanced by using 5D3-OVA. In a previous assay we used BMDCs derived from both WT and MR-/- mice and $CD4^+$ T-cells proliferation was found to be independent of MR (Figure 4.11), this is in agreement with studies reported earlier (Burgdorf et al., 2007). Burgorf et al. (2007) demonstrated that OVA-specific $CD4^+$ T-cells (OTII) proliferation is completely independent from MR and that Ag uptake through MR targets OVA into early endosomes and result in OVA-specific $CD8^+$ T-cell activation. These authors demonstrated that APC use distinct Ag uptake mechanisms and target the Ag to different intracellular compartment specialised for Ag presentation to $CD4^+$ and $CD8^+$ T-cells as shown in figure 4.12.

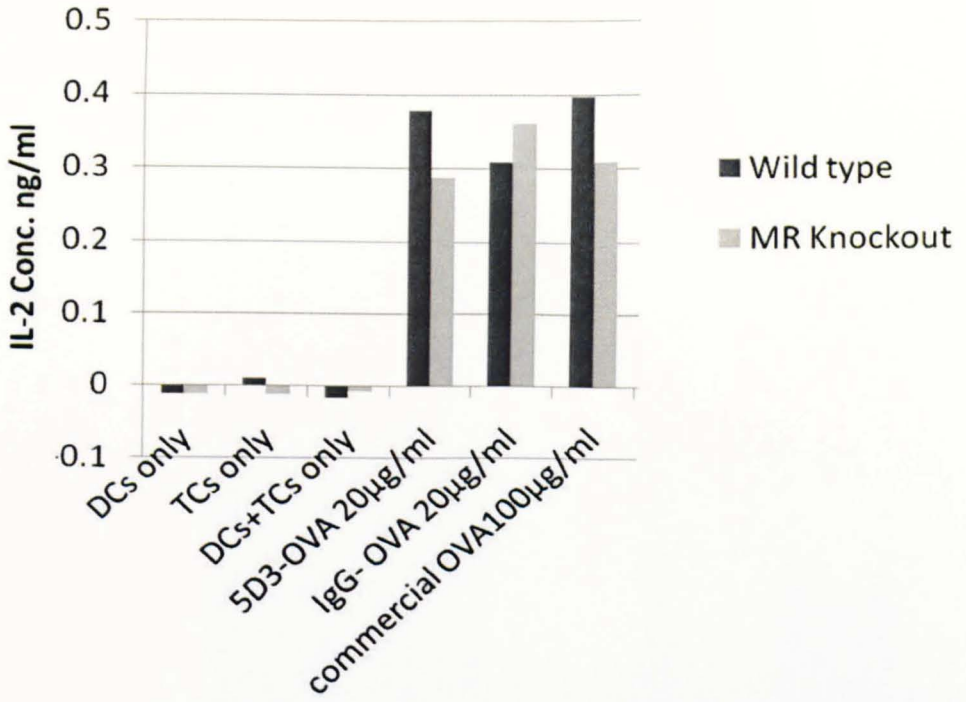


Figure 4.11: $CD4^+$ T-cell (MF2) proliferation induced by BMDC derived from WT and MR-/- mice in response to OVA, 5D3-OVA and IgG1-OVA.

BMDCs obtained from both MR+/+ and MR-/- C57BL/6 mice were incubated with 5D3-OVA, IgG1-OVA and commercial OVA and co-cultured with MF2 Tcells overnight at 37°C and 5% CO_2. The level of OVA specific T-cell proliferation was estimated by measuring the IL-2 cytokine in the supernatant of the co-culture.

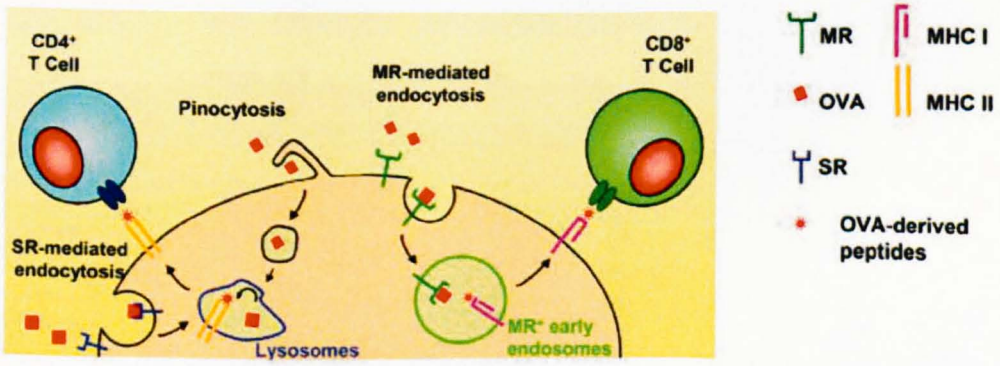


Figure 4.12: Distinct pathways of Ag uptake and presentation by APCs. (Burgdorf et al., 2007). OVA uptake through pinocytosis leads to its presentation through MHC-II pathway and prime $CD4^+$ T-cell while Ag uptake through MR introduces OVA into early endosomes and results in OVA specific $CD8^+$ T-cell activation.

They proved that OVA-specific $CD4^+$ T-cells proliferation is dependent on the pinocytosed OVA using dimethylamiloride (DMA); an inhibitor of pinocytosis that keeps receptor mediated endocytosis intact. OVA-specific $CD4^+$ T-cells proliferation was abrogated in the presence of DMA indicating the importance of pinocytosis in $CD4^+$ T-cells proliferation. DCs uptake Ag contitutively through pinocytosis (independent of MR), which justifies $CD4^+$ T-cells responses obtained by 5D3-OVA and IgG-OVA. However, the same isotype control did not induce $CD4^+$ T-cells proliferation *in vitro* when used with anti-DEC205 Ab to deliver Ag to DCs as reported by Bonifaz et al. and Hawiger et al. (Bonifaz et al., 2002, Hawiger et al., 2001). Tsuji et al. reported that *in vivo* immunisations of anti-MR Abs fused to a tumour Ag can induce tumour specific $CD4^+$ and $CD8^+$ T-cells immunity but not the isotype control (Tsuji et al., 2010). So, either 5D3-OVA Ab lost its ability to bind cellular MR due to degradation as mentioned in section 4.4.1 or genetic manipulation, or the isotype control binds to some endocytic receptor expressed on DCs. In order to address this question a positive control such as DEC205-OVA (Anti DEC205 Ab fused with OVA) should be generated to compare in parallel the effect of antigen targeting through MR (5D3-OVA) and DEC205 (DEC205-OVA) to Ag presentation and T-cells activation.

Moreover, when analysing these results it should be considered that the tools used for Ag targeting through MR in most of the above mentioned studies are mannosylated proteins and MR is not the only receptor specific for mannosylated proteins. Other receptors such as SIGNR1 in mouse and DC-SIGN in humans recognise and are involved in the uptake of high mannose

oligosaccharides (Mitchell et al., 2001). Ags targeted through DC-SIGN are directed to the late endosome or lysosome and are efficiently processed and presented to $CD4^+$ T-cells (Engering et al., 2002).

HEL-specific $CD4^+$ T-cell hybridoma (1C5.1) and BMDCs derived from BALB/C were co-cultured to analyse the capacity of 5D3-HEL, DEC205-HEL and IgG1-HEL to activate Ag specific T-cells. HEL induced a much reduced T-cell proliferation while DEC205-HEL greatly enhanced Ag presentation and $CD4^+$ T-cells activation. This is consistent with the studies performend by Bonifaz et al, who demonstrated that OVA presentation and T-cell proliferation was increased 300 fold when targeted through DEC205 (DEC205-OVA) as compared to unconjugated OVA (Bonifaz et al., 2002). Compared to DEC205-HEL, Ag targeting using 5D3-HEL did not induce T-cells proliferation possibly due to the lack of MR expression by the BMDCs used in this occasion. Moreover, DEC205 differs from MR in Ag presentation as Ag targeted through DEC205 delivers the Ag to both early endosomal compartment and lysosomes for MHC-I and MHC-II restricted Ag presentation respectively (Gurer et al., 2008). On the other hand, MR delivers Ag to early endosomal compartment only for MHC-I restricted presentation. Therefore, the limited T-cells activation observed in response to 5D3-HEL could be independent from MR and might rely on the uptake of 5D3-HEL through pinocytosis (Burgdorf et al., 2007).

Results obtained with the isotype control IgG1-HEL and IgG1-OVA were not consistent with the published data (Bonifaz et al., 2002, Hawiger et al., 2001).

In our hands the isotype control always induced T-cell activation even when used for *in vivo* DNA immunisation (see 4.4.5). We were concerned whether we got the right clones and this was verified by comparing the sequence of the isotype control in our lab with that original sequence obtained by Prof. Michel Nussenweig laboratory (Hawiger et al., 2001). So, the data obtained using chimeric antibodies needs further investigation to clarify these discrepancies. Moreover, it should be taken into consideration that the Fc portion of these Abs although mutated, can still bind to FcγRI and we need to consider the possibility of FcR-mediated uptake/presentation which is dicussed in detail in 5.4.1. FcγRI is high affinity receptor for IgG, it is widely expressed on DCs, macrophages and monocytes (Deo et al., 1997) and antigen uptake through this receptor results in Ag presentation via MHC-I and MHC-II pathway *in vitro* (Wallace et al., 2001, Tacken et al., 2006)

In order to avoid FcR-mediated Ag uptake and presentation single chain Abs carrying OVA (ScFv-OVA) or Fab fragments fused with OVA (Fab-OVA) can be generated. ScFv comprised of the variable region of heavy (V_H) and light chain (V_L) connected by a 15 amino acid linker (Huston et al., 1988). Fab fragments consist of the variable domains and the corresponding first constant domain: VH-CH1 and VL-CL.

4.4.4 Limitations of *in vitro* $CD4^+$ T-cells proliferation assay used to test anti-MR chimeric Abs

Anti-MR chimeric Abs generated in this study have been tested by *in vitro* Ag presentation or co-culture assay which is not a very feasible method to test reagents involved in MR-targeted vaccines due to the following limitations:

Firslty, Anti-MR chimeric Abs such 5D3-OVA and 5D3-HEL are not stable and are highly prone to degradation at room temperature as described in section 4.4.1 and in chapter 7. During Ag presentation assay (*in vitro*) BMDCs and T-cell hybridoma have been co-cultured at 37 °C for 18 hours. Therefore, during the Ag presentation assay anti-MR Abs could degrade rapidly while the isotype control being stable may persist longer to induce T-cells proliferation. In order to circumvent this issue the time of incubation can be reduced to 4-hours as reported by Singh et al. (Singh et al., 2011). Secondly, Jordens et al. have repoted that up to 20% of the newly synthesized MR is secreted into the culture medium in the form of soluble MR (sMR). sMR maintains it binding properties and the high amount of sMR released by DCs can bind directly to 5D3-OVA and 5D3-HEL and therefore could block or reduce their binding to DCs associated MR (Jordens et al., 1999). Moreover, sMR have also been reported by Umut Gazi to be present in FBS used in the culture medium during Ag presentation personal (Gazi et al., personal communication) Moreover, collagen fragments might be present in serum. Martinez et al. reported that collagen fragments are MR ligands (Martinez-Pomares et al., 2006) and therefore they could block MR. This was further confirmed by an endocytosis assay in which MR-mediated endocytosis by BMDCs was abrogated in the presence of collagen (Burgdorf et al., 2010).

Due to these limitations of the *in vitro* Ag presentation assay it will be more appropriate to test anti-MR Abs *in vivo* as reported in the following studies. He et al. described that human anti-MR Ab (B11) fused with OVA (B11-OVA) can induce OVA specific $CD4^+$ and $CD8^+$ T-cell responses when immunized *in*

vivo along with the appropriate adjuvants (He et al., 2007). Similarly, Tsuji et al. demonstrated that B11 fused with a tumour Ag when injected *in vivo* induce anti-tumour $CD4^+$ and $CD8^+$ T-cell responses (Tsuji et al., 2010).

4.4.5 In vivo immunisation with DNA plasmids encoding 5D3-OVA and IgG1-OVA

In vivo immiunisations with DNA encoding MR-OVA and IgG1-OVA were performed to assess their capacity to activate IFNγ producing antigen specific $CD8^+$ T-cells. Mice immunized with plasmid DNA encoding an OVA peptide (Immunobody) were used as positive control. Immunobody was generated by incorporating the SIINFEKL epitope into the CDRH2 or by direct replacement of CDRH3 of human IgG1. When administered, immunobody targets the Ag to APCs through high affinity FcγRs and induce 100 fold higher CTL responses compared to the peptide only (Pudney et al., 2010). DNA immunisations of chimeric Ab were found more effective in inducing T-cell immunity as compared to their protein form as shown in figure 4.9 and 5.10. Enhanced T-cells responses achieved by DNA immunisation (5D3-OVA) as compared to Ab (5D3-TRP-2) could be due to its ability to exploit two pathways of antigen presentation. Firstly, due to the direct Ag presentation through MHC-I when DNA is transfected into local DCs. Secondly, DNA taken up by the somatic cells could be translated and secreted chimeric Abs out of the cells at the site of immunisation and taken up by the local DC through either MR or FcγRI and result in cross presentation of Ag to induce Ag specific $CD8^+$ T-cell responses (Pudney et al., 2010).

However, IgG1-OVA induced OVA specific $CD8^+$ Tcell activation similar to that induced by 5D3-OVA as in the *in vitro* assays and it is possible that this could be caused by low expression of 5D3-OVA and/or effective targeting through FcγR1.

4.4.6 Conclusion

5D3-OVA and IgG1-OVA have been generated and tested tested for their binding to MR. *In vitro* Ag presentation assays using co-cultures of BMDCs and T-cell hybridoma such as B3Z, MF2 and 1C5.1, were performed to investigate the capacity of chimeric Abs to deliver Ags to APCs and activate T-cells. Plasmid DNA encoding 5D3-OVA and IgG-OVA were also immunised *in vivo* and found effective to induce OVA specific $CD8^+$T-cells. However the data obtained by these assays raised questions regarding (i) the validity of results obtained with 5D3-HEL and 5D3-OVA due to their labile nature, (ii) the use of *in vitro* assays when testing MR-mediated Ag presenatation presentation because of the presence of sMR in the supernatant, (iii) the suitability of leaving the Ag throughout the *in vitro* co-culturing assay (iv) the possibility of FcγR-mediated antigen presentation.

5 Antibody-mediated targeting of TRP-2 Ag through MR to induce T-cell immunity

5.1 Introduction:

Conventional methods of cancer treatment involves surgery, radiotherapy and chemotherapy which adversely affect the normal tissues: In order to address this issue many alternate approaches of cancer immunotherapy are being evaluated (Pardoll, 1998). Immunotherapeutic strategies leading to the induction of CTL immunity against the relevant Ag is required to treat cancers (Zeh et al., 1999). Many preclinical studies to develope vaccines against cancer have been performed. Vaccination studies are complicated as it involves the selection of appropriate tumour Ags which are highly immunogenic, vaccination strategies (Ag delivery) as the peptide Ags are unstable when injected *in vivo*, adjuvants and cytokines.

B16 is a murine melanoma, which spreads spontaneously into the host and it is poorly immunogenic but tumour regression could be triggered by immunotherapy with the antigens expressed by the melanoma (Moerch et al., 2000). Xenoenic Ags such as chicken-OVA and bacterial β-Galactosidase are transfected into tumour cells and used often as experimental models in preclinical tumour therapeutic studies (Paglia et al., 1996). These model antigens leads to vigorous immune responses as these are non-self antigens and lack tolerance, while tumour associated Ags are self and the immune responses against them were relatively low due to the presence of tolerance against them. So, in order to develop a real model for immunotherapeutic studies the antigens used should be self and expressed by both the experimental organism and humans, as at the end these studies are going to be translated in humans. Melanocyte differentiation Ags (MDAs) is a group of Ags recognised by T-

cells of melanoma patients associated with MHC-I molecules and includes gp100/pmel-17, tyrosinase, Tyrosinase-related protein 1 (TRP-1)/gp75, Tyrosinase-related protein 2 (TRP-2) and MART-1/Melan-A (Bronte et al., 2000). To break tolerance and induce active immunity against these different tumour-associated self antigens, different vaccination strategies have been adapted which include immunisation with: antigen (peptide)-pulsed DC, naked DNA using gene gun method, purified antigens mixed with endotoxins and antibodies fused with Ag to target DC receptors (Bellone et al., 2000).

TRP-1/gp75 Ag expressed in insecT-cells or when trasfected as DNA led to induction of immunity and the eradication of the melanoma but it also caused depigmentation of the skin (Vitiligo) due to autoimmune response and destruction of some normal melanocytes sharing TRP-1/gp75 Ag expression (Overwijk et al., 1999). TRP-2 antigen is expressed by normal and malignant melanocytes in both mouse and human (Wang et al., 1996, Bloom et al., 1997) and is ideal for vaccination as TRP-2 specific T-cells have been identified in melanoma patients which show that it is an immunogenic Ag. Immunisation with plasmid encoding murine TRP-2 antigen induced CTL responses in mice which led to the eradication of B-16 melanoma cells but did not cause vitiligo which makes this Ag a good candidate for vaccination (Wang et al., 1996).

MR-specific antibodies fused to cancer antigen NY-ESO-1 have been used to target dendritic cells specifically and result in the induction of antigen-specific $CD8^+$ and $CD4^+$ T-cells which makes it a promising approach to develop strong immunity in cancer. DCs targeting through MR in the presence of an

appropriate ajuvant leads to cross presentation and the induction of powerful CTL immunity required for the immunotherapy of cancer (Tsuji et al., 2010).

In this study, MR specific mAb (5D3 and 6C3) and isotype control IgG2a (Martinez-Pomares et al., 2003) have been conjugated covalently with TRP-2, a MHC-I restricted melanoma Ag. Binding of antibodies to MR before and after conjugation have been analysed by ELISA in order to assess the effect of chemical coupling on the binding, moreover MR-mediated internalization have also been assessed by using CHO cells expressing MR. TRP-2 conjugates were injected subcutaneously into mice in the presence of MPLA and CpG as adjuvants. After two booster doses mice were sacrificed and spleens were collected and TRP-2 specific T-cells secreting IFNγ were counted by ELISPOT.

5.2 Materials and methods:

5.2.1 Mice

C57BL/6 mice were bred at BMSU as described in section 2.1.1.

5.2.2 Synthesis of Trp-2 peptide and its deprotection

Tyrosinase related protein 2 (TRP-2) (SVYDFFVWL) (Bloom et al., 1997), was synthesized at The Biopolymer Synthesis and Analysis unit, Nottingham University with an added cysteine at the C-terminus of the petide to generate a free sulfhydryl group to facilitate the chemical coupling to the anti-MR monoclonal antibodies and isotype control. A biotin molecule was added at the C-terminus of the peptide using a PEG (Polyethylene glycol) spacer (Pierce

Biotechnology) to track the labelling. The crude TRP-2 peptide was deprotected by overnight mild acid treatment. After deprotection the TRP-2 peptide was purified by high performance liquid chromatography (HPLC) and analysed by Q-TOF mass spectrum.

5.2.3 Chemical coupling of TRP-2 peptide to anti-MR and isotype-matched control monoclonal antibodies (mAbs)

Dr. Weng Chan helped in the chemical coupling of TRP-2 peptide with anti-MR mABs (School of Pharmacy). Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) (Pierce Biotechnology) was used to activate the monoclonal anti-MR antibodies 5D3 and 6C3 and their isotype control IgG2a (Martinez-Pomares et al., 2003). The mAbs were treated with Sulfo-SMCC at a 10:1 molar ratio for 30 minutes to generate sulfo-reactive groups. The activated mAbs were purified by size exclusion chromatography, using D-salt dextran desalting column (Pierce Biotechnology) following the manfacturere's instructions. The mAbs were eluted in PBS and quantified by BCA protein quantification assay (Pierce Biotechnology).

The TRP-2 peptide containing the sulfhydryl group was freshly prepared by dissolving it in 20% DMSO-H_2O at concentration of 1 mg/ml. The solution containing TRP-2 peptide was mixed with the monoclonal antibodies at a 5:1 molar ratio and incubated at 37°C for 1 hour. The mix was further incubated overnight at 4°C for better coupling (Figure 5.1). Antibody-peptide conjugate was purified by protein G chromatography following the protocol described in section 2.2.1. Purified conjugates were dialysed against PBS using Dialysis

cassettes (Pierce Biotechnology). The dialysed conjugates were quantified by BCA protein quantification assay and the extent of biotinylation (number of peptide coupled to mAb) was determined by using the Fluoreporter kit (Invitrogen) following the instructions provided by manufacturer.

5.2.4 *In vitro* TRP-2 specific antigen presentation assay

5.2.4.1 Harvesting the bone marrow and generation of BMDCs

GM-CSF induced bone marrow derived DCs were generated following the protocol described in section 2.3.1.

5.2.4.2 Generation of TRP-2 specific T-cells

TRP-2 specific T-cells were generated by Dr. Victoria at City Hospital, Department of Clinical Oncology, University of Nottingham.

5.2.4.3 Co-culture assay

DCs were incubated with 50μg of conjugates overnight and then co-cultured with TRP-2 specific CTLs at City Hospital, Clinical Oncology department. ELISPOT assay was performed in order to look at the ability of TRP2-specific CTLs to recognize TRP-2 presented by these DCs and their activation measured by ELISPOT.

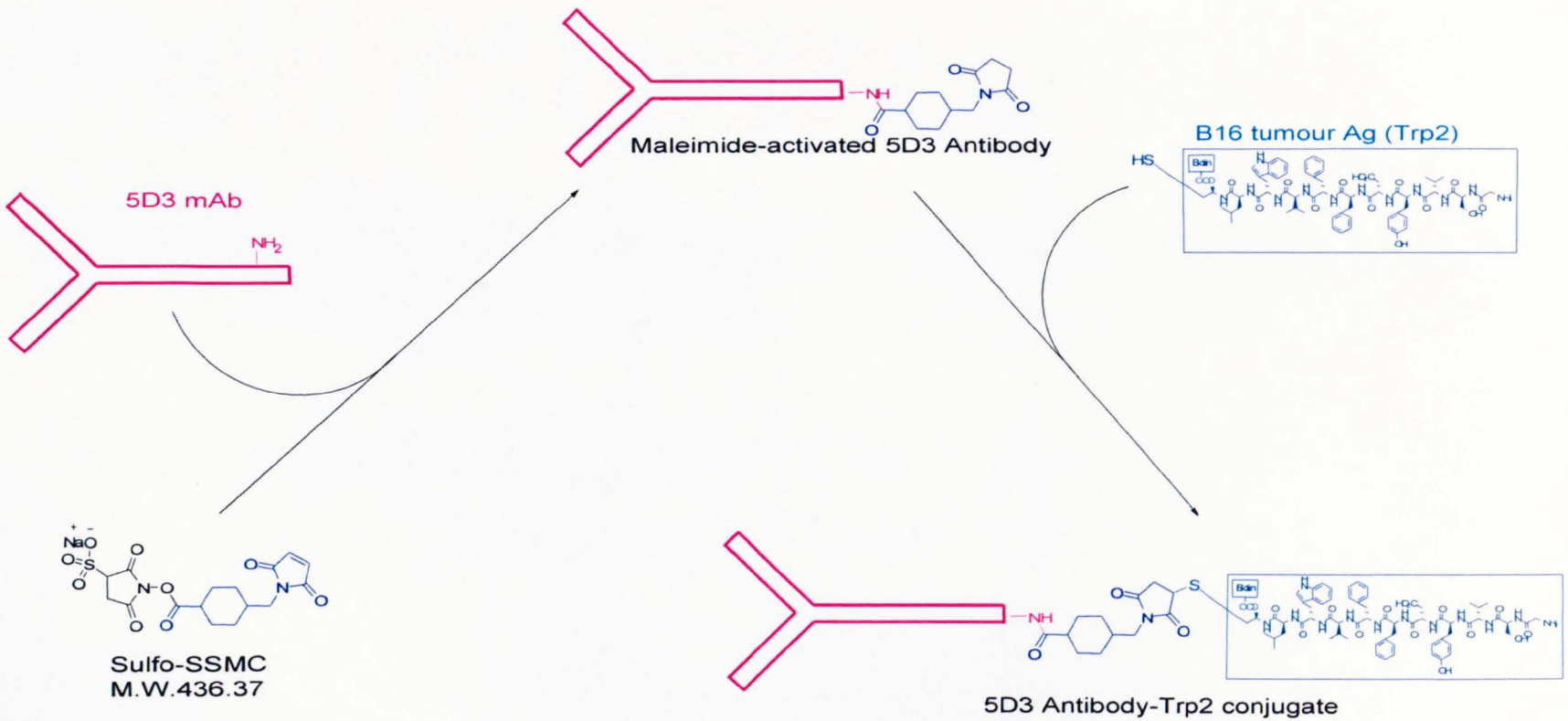


Figure 5.1: Schematic diadram showing the mechanism of chemical coupling of TRP-2 peptide to 5D3 and other IgG molecules.

5.2.5 Immunisation studies

Mice were divided into four groups for vaccination experiments. Each mouse was injected subcutaneously at the base of the tail with 10μg of anti-MR mAbs or their isotype-matched control covalently coupled with TRP-2 along with 6μg of MPLA as an adjuvant. One group was immunised with IB DNA-TRP-2 plasmid by gene gun method and it was used as a positive control (Metheringham et al., 2009). Plasmid DNA carrying the TRP-2 gene fragment was coated on 1.0 μm gold particles following the manufacturer's instructions (Biorad, Hemel Hempstead). DNA particles were administered intardermally into the shaved abdomen of the mice by using Helios Gene Gun (Biorad) and each mouse was immunised with 1.0μg of DNA per immunisation. After this primary immunisation, secondary and tertiary immunisations were done one week apart to act as booster doses. One day after the tertiary immunisation, mice were sacrifice and their blood, lymph nodes and spleens were collected in order to analyse the immune response against TRP-2 antigen and ratIgG.

In-vivo immunisations were repeated by following the same parameters except MPLA (3μg) and CpG (3μg) were used as adjuvants instead of using only MPLA, during second immunisation.

5.2.6 ELISA to detect humoral response against rat-IgG

Serum was collected from the blood of immunised mice and analysed for humoral response in the form of anti-rat IgG which was detected by using ELISA. MAXISORP 96 well flat bottomed plates (Nunc Cat no 439454) were coated with 10 μg/ml of rat IgG. The coated plates were incubated overnight at 4°C. The next day the plate was washed three times with PBS and blocked with

3% BSA in PBS for an hour at 37°C. Then, it was washed 3X with PBS-0.1% (v/v) Tween 20 and incubated with appropriate dilutions of sera for an hour at room temperature. Then the plate was incubated with donkey anti-mouse IgG-AP diluted 1:2000 (Jackson Immunoresearch laboratories) for one hour. The plate was again washed 3 times with PBS 0.1% (V/V) Tween20, followed by 2 times with AP developing buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2.6H_2O$ pH 9.5), before being incubated with 1mg/ml of p-nitrophenyl phosphate substrate (SIGMA). The OD absorbance was measured at 405nm on a 96-well plate reader (MultiskanEX; Labsystem).

5.2.7 ELISPOT to detect TRP-2 specific T-cells producing IFNγ

ELISPOT assay was used to determine the frequency of peptide-specific IFNγ-secreting T-cells in murine splenocytes and in *in vtro* assay, using mouse IFNγ ELISPOT kit (Mabtech) and following the protocol described in section 4.2.9.

5.3 Results

5.3.1 Preparation of Anti-MR-TRP-2 conjugates and their characterization

The TRP-2 peptide was synthesized and purified by high performance liquid chromatography (HPLC). The purity of the molecule was found more than 70% as shown in the Figure 5.2. The main HPLC peak was analysed using Q-TOF mass spectrum and two molecules of molecular weight 1704.829 Dalton and 1750.815 Dalton were observed respectively. The increase in mass in the 1750.815 peak was found due to the addition of *tert*-Butyloxycarbobnyl (BOC) molecule which is used to protect reactive amines during the synthesis of peptides (Figure 5.3). The TRP-2 peptide was then deprotected by overnight treatment with mild acid. After deprotection the peptide was lyophilised again and analysed by Q-TOF. A single peak of mass 1706.81 was observed after deprotection (Figure 5.4). Deprotected TRP-2 peptide was then chemically coupled to the anti-MR mAbs and their isotype control by covalent bond. After, chemical coupling the conjugates were purified by affinity chromatography and their degree of labelling was calculated by using a fluoreporter kit. The degree of labelling of 5D3-TRP2, 6C3-TRP2 and IgG2a-TRP2 was found to be 1.2, 1.5 and 1.7 peptides per molecule of IgG respectively.

The purified conjugates were analysed by SDS-PAGE and a shift in the size of mAbs was observed as shown in Fig.5.5.A. In order to confirm the presence of biotinylated peptides western blotting using streptavidin-peroxidase conjugate was performed and clear bands showed the successful conjugation. On the

basis of band intensity the degree of labelling was confirmed as calculated by fluoreporter kit. IgG2a-TRP2 showed highest intensity then 6C3-TRP-2 and 5D3-TRP-2 showed the least intense band on western blot Fig.5.6.

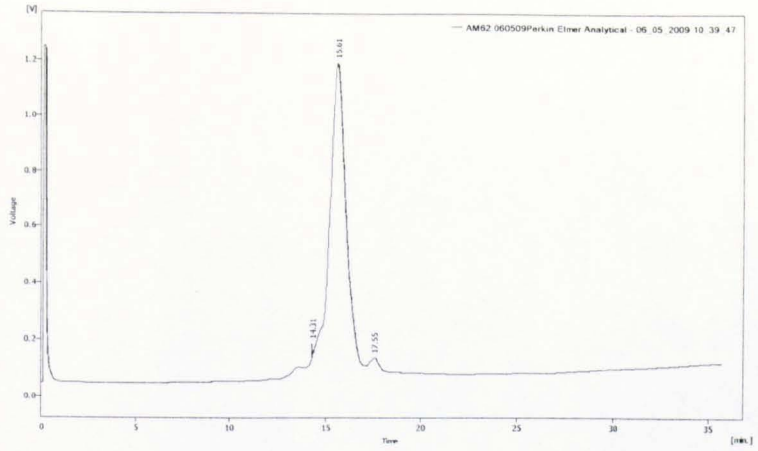


Figure 5.2: Reverse-phase chromatogram of TRP-2 peptide synthesized at the Biopolymer Synthesis and Analysis Unit, Nottingham University.

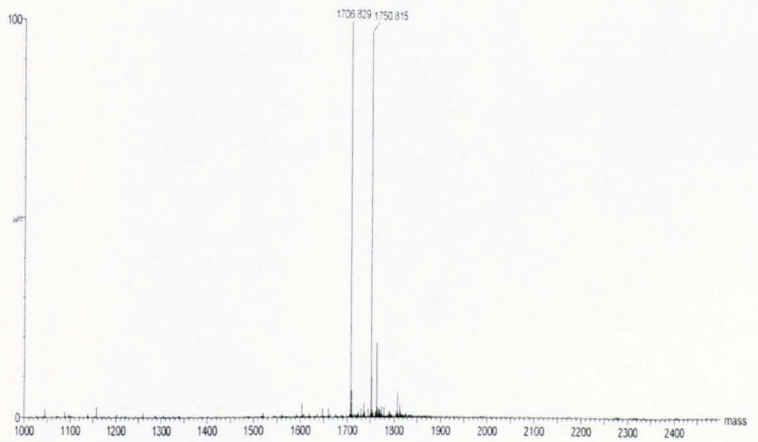


Figure 5.3: Chromatogram obtained by the Q-TOF mass spectrum analysis of main HPLC peak shown in figure 5.1.

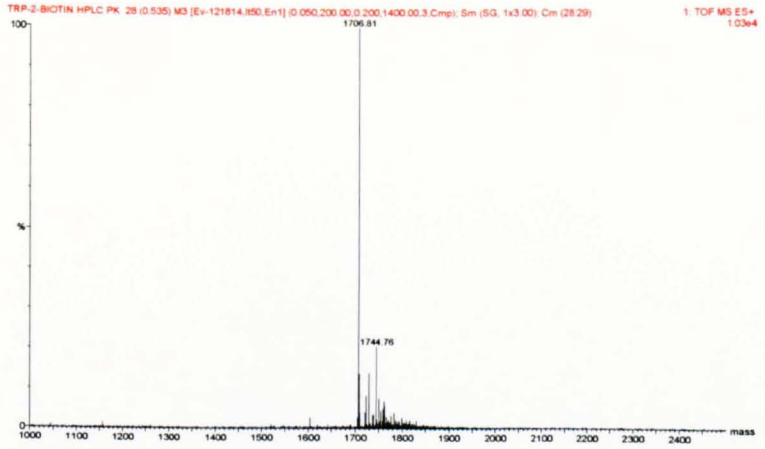


Figure 5.4: Q-TOF mass spectrum analysis of the TRP-2 peptide after its deprotection.

The crude TRP-2 peptide was deprotected by overnight treatment with mild acid and purified using HPLC by the peptide synthesis facility. Q-TOF mass spectrum analysis was performed to confirm the deprotection of TRP-2 peptide.

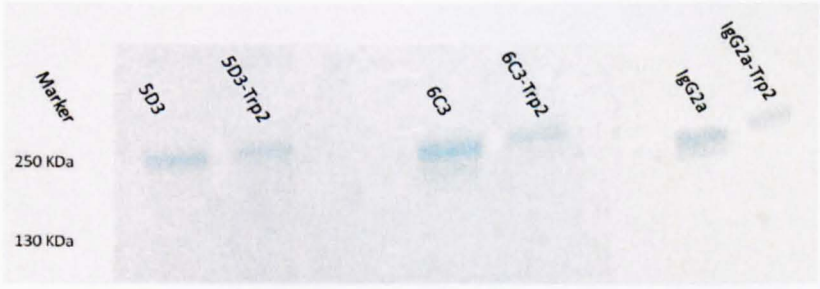


Figure 5.5: SDS-PAGE analysis of anti-MR monoclonal 5D3 and 6C3 and IgG2a before and after chemical coupling.

1.5µg of the mAbs was loaded on a 6% SDS-PAGE and proteins were visualised by gel staining with blue safe for one hour and destained with distilled water overnight.

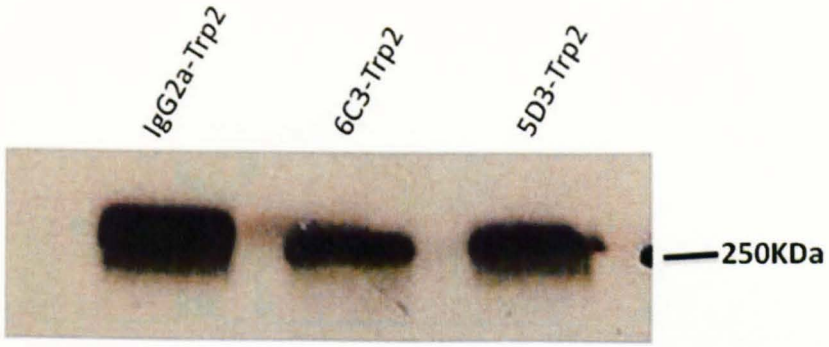


Figure 5.6: Western Blot to confirm chemical coupling of biotinylated TRP-2 peptide to the monoclonal antibodies.

150 ng of conjugates were electrophoresed on 6% SDS-PAGE and transferred to the nitrocellulose membrane. The nitrocellulose membrane was blocked with 4% BSA in PBS and the biotin molecule in TRP-2 peptide coupled to the mAbs was detected using streptavidin-HRP.

5.3.2 Analysis of the binding of anti-MR conjugates to recombinant MR-constructs

The binding ability of the mAbs after chemical coupling was determined by ELISA using the MR-construct CTLD4-7-Fc as Ag. After chemical coupling anti-MR conjugates showed binding to MR domain CTLD4-7Fc. The chemical coupling seemed to reduce the binding ability of both anti-MR mAb such 5D3 and 6C3 but its affect was more on the binding of 6C3 as shown in Figure 5.7. The isotype-matched control of the anti-MR mAbs did not showed any binding before and after chemical coupling.

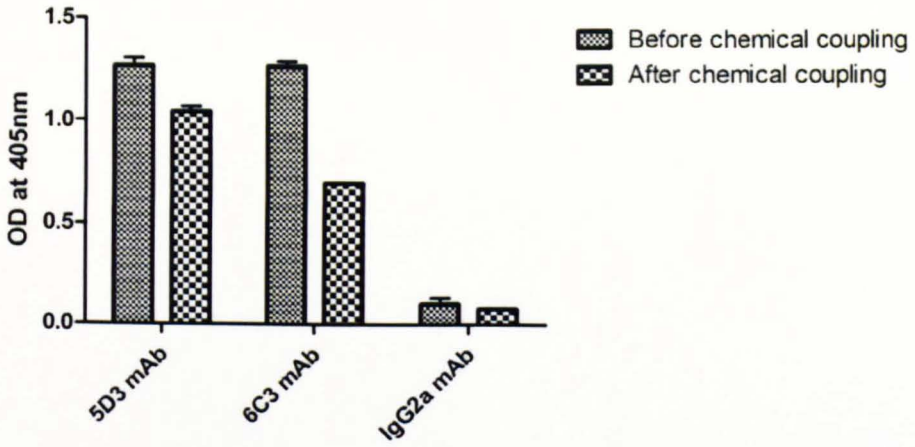


Figure 5.7: Binding of anti-MR monoclonal antibodies to CTLD4-7-Fc before and after chemical coupling of TRP-2 peptide.

The plate was coated with 5μg/ml of CTLD4-7Fc, which is recognised by anti-MR mAbs, followed by the addition of 1μg/ml anti-MR mAbs and anti-rat-AP conjugated antibody as a secondary antibody and the level of binding was measured by taking absorbance at 405nm after the addition of 1mg/ml of p-nitrophenyl phosphate substrate.

5.3.3 MR-mediated endocytosis of different TRP-2 conjugates

In order to analyze the MR mediated internalization of anti-MR conjugates CHO cell line expressing MR (CHO-MR) was used and WT CHO was used as a negative control. CHO and CHO-MR were incubated with anti-MR mAbs and the level of internalization was assessed by using Alexa488-labelled anti-rat IgG as secondary Ab and flow cytometric analysis. Anti-MR mAb 6C3-TRP2 showed better internalization as compared to 5D3-TRP2 (both shown in blue histograms) as shown. The isotype control (shown in red histogram) did not show any endocytosis as shown in Figure 5.8.

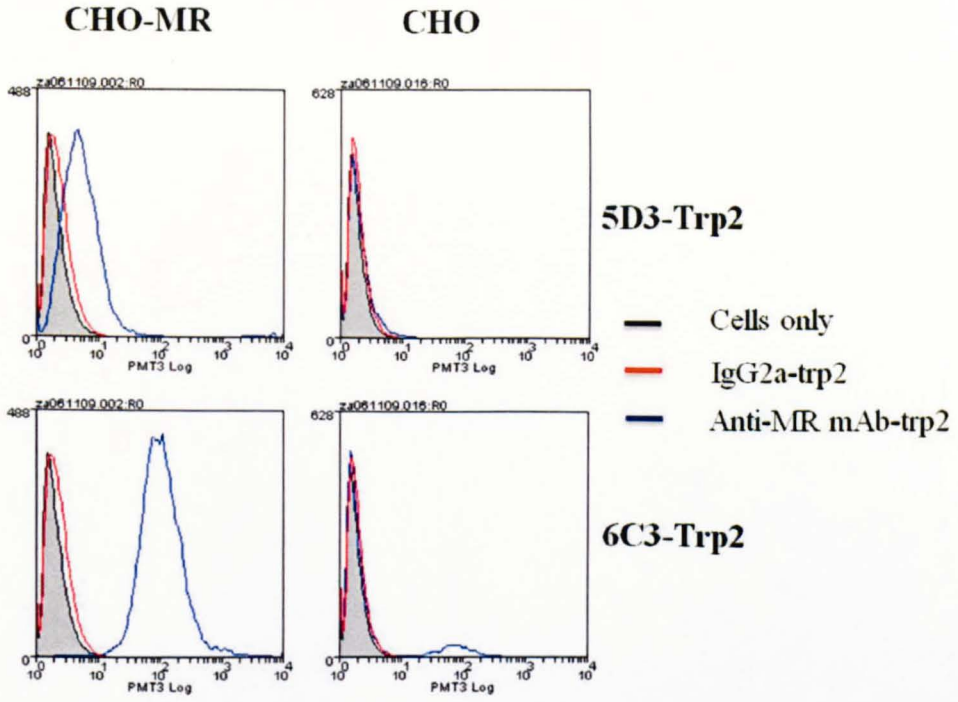


Figure 5.8: Histograms showing MR-mediated internalization of different anti-MR mAbs conjugates.

CHO and CHO-MR cells were incubated with 20μg/ml of anti-MR-TRP2 conjugates for 30 minutes followed by permeabilization of the cells and incubation with Alexa488 labelled anti-rat IgG.

5.3.4 Analysis of the ability of anti-MR conjugates to deliver TRP-2 to DCs and activate TRP-2 specific T-cells *in vitro*.

The ability of conjugates to activate TRP-2 specific T-cell response was assessed by co-culture of BMDCs fed with anti-MR-TRP-2 conjugates with TRP-2 specific T-cells cultured by Dr. Victoria Pudney (City Hospital, Clinical Oncology department). Activation of, IFN-γ secreting TRP-2 specific T-cells was assessed by using ELISPOT. TRP-2 specific CTLs and DCs alone were considered as the negative controls and showed a negligible number of cells expressing IFN-γ as shown in Figure 5.9. In the absence of TRP-2 peptide RMAS cells (A non professional antigen presenting cell line which is used as a

positive control in the presence of TRP-2 peptide) did not show any T-cell activation capacity, but it showed very good T-cell activation in the presence of TRP-2 peptide which indicates that T-cell activation in this assay is TRP-2 specific (Figure 5.9). Generally, conjugates did not showed very good CTL activation potential except 6C3-TRP2 which showed marginally a better response as shown in the Figure 5.9.

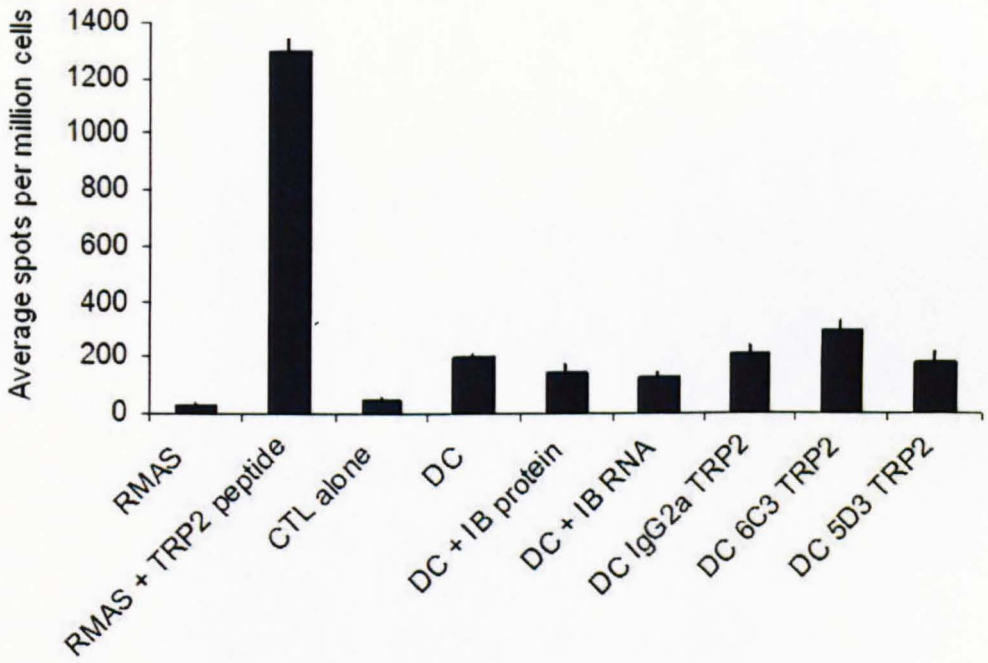


Figure 5.9: ELISPOT assay showing the number of IFN-γ secreting TRP-2 specific T-cells when cocultured with BMDCs in response to anti-MR conjugates *in vitro*.

Mouse BMDCs fed with anti-MR conjugates (IgGs) were co-cultured with a TRP-2 specific T-cell line overnight. The number of IFN-γ secreting TRP-2 specific T-cells was counted from the culture by using ELISPOT. RMAS is a non professional antigen presenting cell line which is used as a positive control in the presence of TRP-2 peptide. Graph shows mean spots/million splenocytes plus standard deviation (SD).

5.3.5 Immunisation with anti-MR conjugates using MPLA only and mix of MPLA and CpG as adjuvants.

5.3.5.1 Analysis of TRP-2-specific T-cells activation

TRP-2 was synthesized and conjugated to anti-MR mAbs in order to target TRP-2 to APCs and induce TRP-2 specific $CD8^+$ Tcells. DNA plasmid encoding TRP-2 Ag delivered using a gene gun method was used as positive control. Initially, immunisations were performed by using MPLA only as an adjuvant but it didn't show promising results as the number of activated TRP-2 specific T-cells was found to be less than in the case of DNA immunisation which was used as a positive control (Data not shown). Immunisations were repeated using MPLA and CpG as adjuvants to achieve innate activation through TRIF and MyD88 signalling. CpG motifs helps to recruit TAP to the early endosome and to cross-present Ag and induce cell mediated immunity (Burgdorf et al., 2008). Moreover, MHC-II loading organelles require TLR4/MyD88 signalling to load phagocytosed Ag to MHC-II molecules and to induce humoral immunity (Blander and Medzhitov, 2006).

Splenocytes from the immunised mice were collected and IFN-γ secreting TRP-2 specific T-cells were counted using ELISPOT. Mice immunized with 5D3-TRP-2 conjugate showed highest frequency of activated TRP-2 specific T-cells (Figure 5.10 B) as compared to mice immunised with plasmid DNA encoding TRP-2 peptide (Positive control). Mice immunised with IgG2a-TRP-2 (Figure 5.10 C) showed relatively less frequency when cultured in low peptide concentration but at higher peptide concentration the frequency was even more than positive control (Figure 5.10 A).

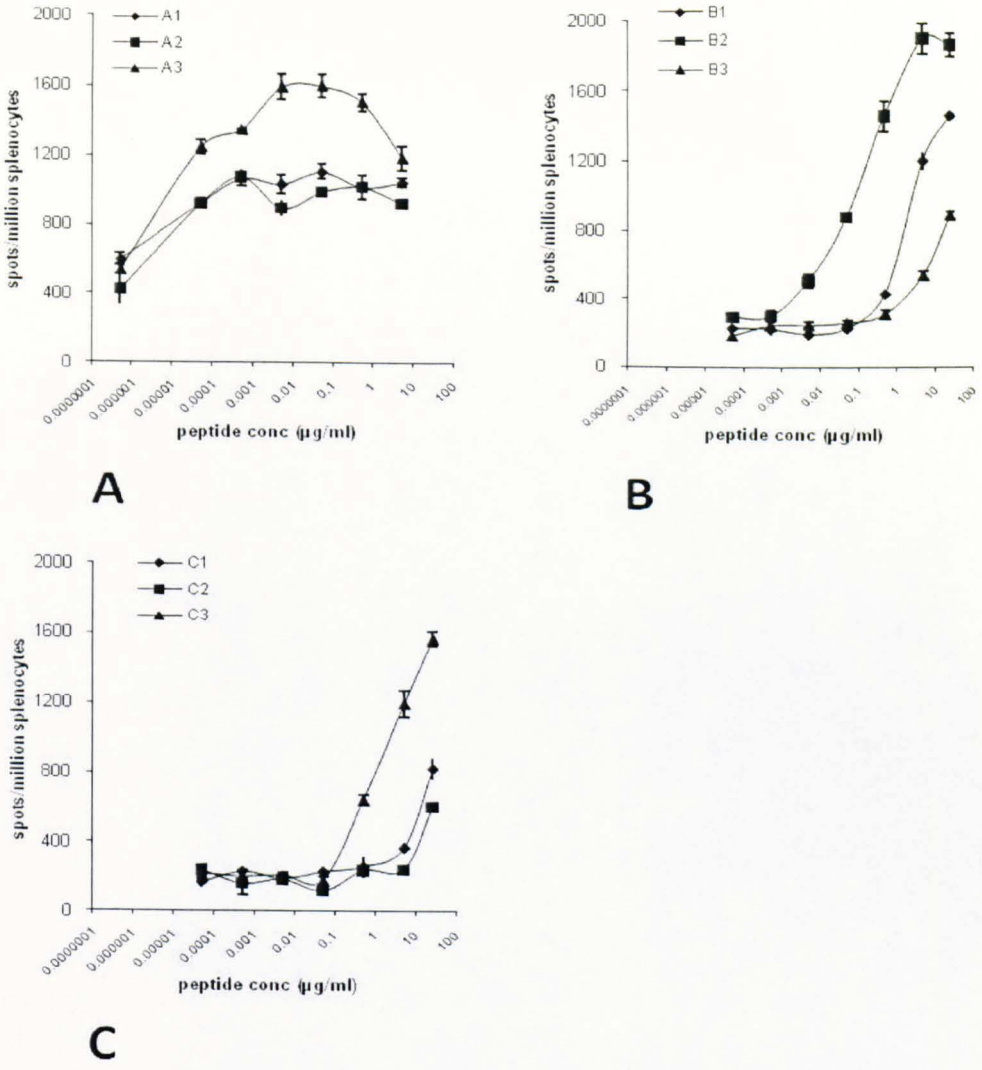


Figure 5.10: Analysis of TRP-2 specific T-cell responses in mice immunised with anti-MR conjugates along with MPLA and CpG as an adjuvant.

After three vaccinations, splenocytes were collected from the different groups of mice immunised with the TRP-2-conjugates and cultured in the presence of different concentrations of the TRP peptide. The number of IFN-γ secreting TRP-2 specific T-cells was counted from the culture by using ELISPOT assay in which each spot indicated the presence of IFN-γ secreting TRP-2 specific T-cell (A) Mice immunised with the TRP-2 encoding DNA (B) Mice immunised with 5D3-TRP-2-conjugate and (C) Mice immunised with IgG2a-TRP-2 conjugate. Graphs show mean spots/million splenocytes plus standard deviation (SD).

5.3.5.2 Anti-rat IgG Ab response in animals immunised with Rat-IgG-TRP-2 conjugates.

The antibody response against anti-MR conjugates was measured by ELISA in the sera from mice immunised with TRP-2-mAb conjugates using MPLA as adjuvant (Data not shown) or using a mixture of MPLA and CpG (Figure 5.11). A good anti-rat antibody response was observed in the mice immunised with 5D3-TRP-2, 6C3-TRP-2 and IgG2a-TRP-2 proved that these conjugates could induce B-cell immunityin the presence of adjuvants. The fourth group immunised with plasmid DNA did not showed any anti-rat antibody (anti-rat IgG) response which showed that the antibody response was specific against rat IgG. Second time mice were immunised with TRP-2 conjugates in the presence of MPLA and CpG as adjuvants similar pattern of anti-rat antibody response was observed.

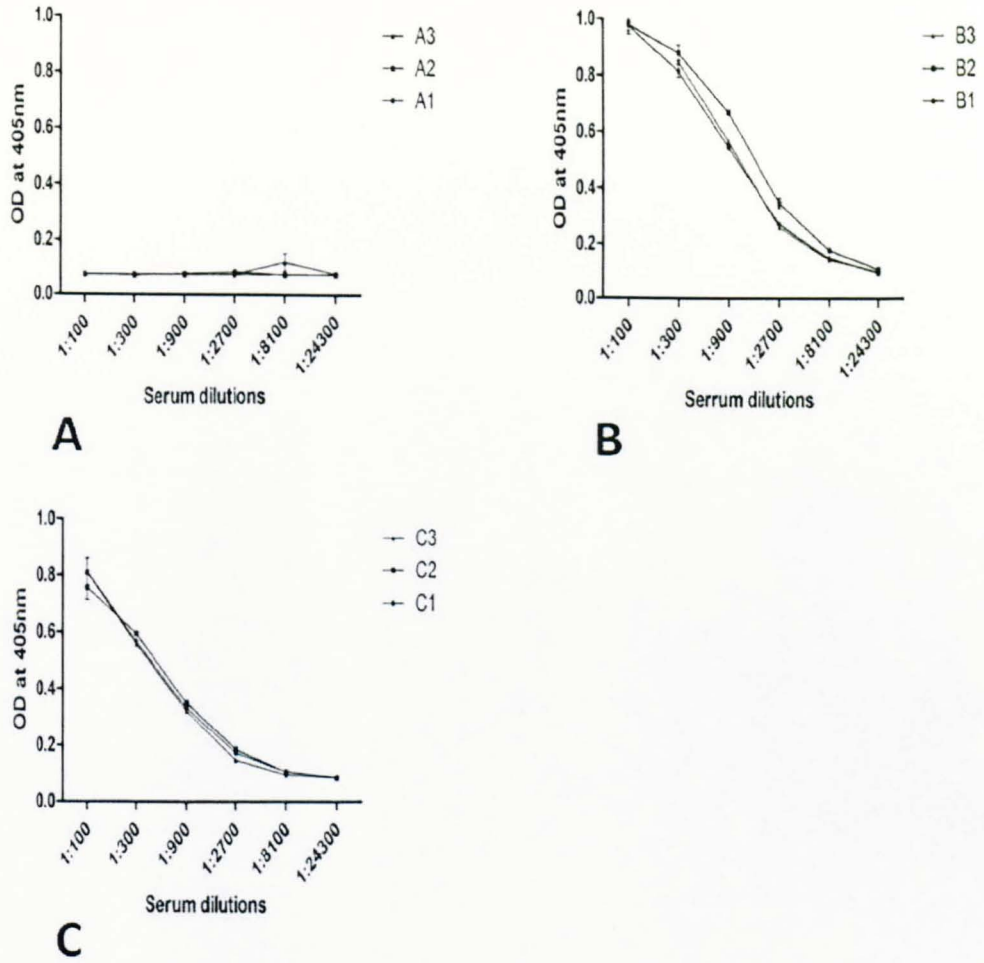


Figure 5.11: Measurement of anti-rat IgG humoral responses in mice immunised with rat-IgG using ELISA.

Serum was collected from the blood of mice immunised with TRP-2 plasmid (A), 5D3-TRP-2 (B), and IgG2a-TRP-2 (C) and diluted 1:100 to 1:24300 using PBS. The ELISA plate was coated with 10µg/ml of rat IgG and the amount of anti-rat antibodies in different dilutions of the serum was measured by measuring OD at 405 nm after adding the alkaline phosphatase substrate to the plates coated with alkaline phosphatase conjugated anti-mouse antibody.

5.4 Discussion:

In this study we conjugated the peptide TRP-2, a melanoma-associated CD8 epitope (SVYDFFVWL) (Bloom et al., 1997) to MR-specific mAbs and an isotype control rat-IgG2a to investigate the potential of these conjugates to target APCs through MR and activate TRP-2 specific $CD8^+$ T-cells which are required to treat cancer. McKenzie et al. reported immunisation with these MR-specific mAbs in combination with endotoxin can target MR^+ DCs in T-cell areas of draining LNs and induce enhanced humoral reponses *in vivo*. The humoral responses were completely abrogated in MR-/- mice indicating the role of MR to promote B cell responses (McKenzie et al., 2007).

A cysteine residue and a biotin molecule were added at the C-terminus of the peptide to generate a free sulfhydryl group to facilitate the chemical coupling and to track the labelling respectively. Antibody-peptide conjugates were successfully generated. The purified conjugates were analysed by SDS-PAGE and a shift in the size of TRP-2 conjugated mAbs was observed which indicated that the conjugation was successful and this was further confirmed by westerb blotting using streptavidin-peroxidase (Figure 5.5 and 5.6). The amount of TRP-2 peptide conjugated to each IgG molecule was calculated by using a fluoreporter kit. The degree of labelling of 5D3-TRP2, 6C3-TRP2 and IgG2a-TRP2 was found to be 1.2, 1.5 and 1.7 peptides per molecule of IgG respectively. The same chemical coupling method has been employed in a study in which a CD8 epitope of OVA was covalently linked to anti-DNGR-1 Ab (Sancho et al., 2008). DNGR-1 is a DC-restricted C-type lectin. It is an endocytic receptor and antigen targeting using a mAb-Ag conjugate together

with adjuvant induced tumour-specific immunity and protected mice challenged with tumour cells (B16-OVA-GFP melanoma cells) (Sancho et al., 2008).

After chemical coupling the binding properties of anti-MR mAbs to the MR domains CTLD4-7 was analysed, and reduced binding was observed. Stable transductants expressing full length MR (CHO-MR) were employed to assess the endocytosis of anti-MR mAbs after chemical coupling of TRP-2 peptide. 6C3-TRP2 was endocytosed more efficiently as compared to 5D3-TRP-2 in agreement with previous studies in which it was shown that 6C3 mAb has more internalization capacity than 5D3 (Martinez-Pomares et al., 2003). Changes in endocytosis could be due to the chemical coupling or differential binding capacity of these mAbs to MR. MR is a recycling endocytic receptor and during recycling it releases its ligands inside the endosome, a process likely dependent on the quality of the interaction between MR and its ligands. As 5D3 binds more efficiently to CTLD4-7 than 6C3 and the release rate of 5D3 is also low as compared to 6C3 (see figure 3.4 and 3.5), 6C3 could be released into the endosome more efficiently than 5D3 which would facilitate receptor recycling and increase the level of internalization.

5.4.1 *In vivo* immunisation with anti-MR-TRP2 conjugates activates TRP-2 specific INFγ secreting T-cells

Antigen delivered to APCs through MR using MR-specific Ab along with appropriate adjuvants leads to the cross presentation and activation of $CD8^+$ T-cells(Tsuji et al., 2010, Ramakrishna et al., 2004, He et al., 2007). In order to

assess the potential of TRP-2 conjugates to deliver antigen and activate CD8+ T-cells, mice were immunized subcutaneously with these conjugates together with adjuvant. In the presence of only MPLA no significant T-cell activation was achieved. MPLA binds TLR-4 and signals through the TRIF mediated pathway (Mata-Haro et al., 2007). TLR-4 engagement by LPS that signals through TRIF and MyD88 is essential for the maturation and migration of local DCs loaded with anti-MR Ab to local lymph nodes (McKenzie et al., 2007). Moreover MyD88 signalling recruits TAP to early endosomes which is believed to be involved in the transfer of peptide and allows loading on the MHC-I molecule in the early endosome leading to cross presentation (Burgdorf et al., 2008). Therefore, the immunisations were repeated using both MPLA and CpG as adjuvants as CpG signals through MyD88.

Mice immunized with plasmid DNA encoding TRP-2 peptide (Immunobody) were used as positive control. Immunobody was generated by incorporating the TRP-2 epitope into the CDRH2 of human IgG1. When immunized, immunobody targets the Ag to APCs through high affinity FcγRs and induce 100 fold higher CTL responses compared to the peptide (Pudney et al., 2010). In our study, Abs-TRP2 conjugates were not as good as the immunobody to induce TRP-2 specific $CD8^+$ T-cells. Enhanced T-cells responses achieved by immunobody are due to to its ability to exploit two pathways of antigen presentation. Firstly, due to direct TRP-2 presentation through MHC-I when the immunobody is transfected into local DC present in the skin resulting in the activation of $CD8^+$ T-cells. Secondly, DNA/immunobody taken up by the somatic cells could be translated and secreted out of the cells at the site of

immunisation and taken up by the local DC through FcγR and result in cross presentation (Pudney et al., 2010) however, the comparision would be difficult because effective doses of DNA and its protein counterpart might be very different . Chimeric Abs induced relatively low T-cells activation as they rely only on one pathway of Ag presentaion by Ag targeting to APCs, however DNA immunisations exploit both APC and non-APC for Ag expression and presentation. Cho et al. analysed the contribution of APC and non APC in DNA immunisations by using tissue and cell (APC) specific promoters. They demostrated that DNA immunised into non APC induced more rapid and vigorous $CD8^+$ T-cells proliferation as compared to APC. Firstly, due to relatively more numbers of non APC and secondly, due to prolonged expression of DNA in non APC as compared to APC (Cho et al., 2001).

In this particular study, the mice immunized with 5D3-TRP-2 induced higher frequency of TRP-2 specific $CD8^+$ T-cells activation at relatively lower concentration of the peptide as compared to the isotype control (IgG2a-TRP-2) which indicated that rat IgG2a could also improve antigen uptake and activation of CD8+ T-cells (Figure 5.10). One possible explanation for this is the targeting of FcγR by rat IgG2a. The receptors specific for the Fc region of IgG (FcγRs) are expressed by many immune cells and are critical for the promotion and regulation of immune responses. These molecules were first described on macrophages followed by B cells and dendritic cells. Murine DCs predominantly express FcγRI, FcγRIIB and FcγRIII (Nimmerjahn and Ravetch, 2008). FcγRs can be activating receptors and these include high affinity receptor such as FcγRI and low affinity recetor such as FcγRIII. However,

FcγRIIB is an inhibitory receptor (Smith and Clatworthy, 2010). IgG molecules complexed with Ag, immune-complexes (IC) have been employed to target Ags to DC through FcγRs and to induce Ag specific immune responses.

ICs can either promote or suppress immune response depending on their interaction with FcγRs. ICs engagement through activating FcγRs with an immunereceptor-tyrosine based activation motif (ITAM) leads to cellular activation. On the otherhand ICs engaged through the inhibitory FcγRIIB signal through immunereceptor-tyrosine based inhibition motif (ITIM) that inhibit ITAM mediated activation signals (Kalergis and Ravetch, 2002). Regnault et al. reported that Ag uptake by DCs through FcγRs is 100 times more efficient as compared to the soluble Ags and uptake through FcγRs induce DCs maturation effectively (Regnault et al., 1999). FcγRs-mediated ICs intaernalization results in MHC-II as well as MHC-I restricted Ag presentation or cross presentation of Ags therefore priming both $CD4^+$ and $CD8^+$ T-cell responses (Regnault et al., 1999).

Akiyana et al. demonstrated that mouse IgG1 ang IgG2a molecules complexed with OVA are able to target DCs through FcγRs and results in MHC-I and MHC-II specific Ag presentation *in vivo* (Akiyama et al., 2003). The mAbs-TRP-2 conjugates are rat-IgG2a which are known to have a very strong affinity for activating Fc receptor (Getahun et al., 2004) and it would be expected that antigen delivery through this receptor might lead to cross-presentation and activation of antigen specific $CD8^+$ T-cells. Murai et al. reported that the engagement of MR with its ligand activates or enhances FcγR-mediated

phagocytosis of mouse IgG potentially by increasing the number of FcγRs or by the enhancement of FcγR function (Murai et al., 1995, Chavele et al., 2010). Therefore, the enhanced adaptive immune response obtained in terms of TRP-2 specific CD8+ T-cells activation by mice immunized with 5D3-TRP-2 could be caused by Ag delivery through MR and FcγR and by the additive effect of MR enhancing FcγR function.

5.4.2 *In vivo* immunisation of anti-MR-TRP2 conjugates induces humoral immunity

MR is an endocytic receptor and Ab-mediated Ag targeting through this receptor is known to induce Ag-specific humoral immunity (McKenzie et al., 2007). In order to analyse whether MR-specific rat IgGs-TRP2 can induce anti-rat IgG humoral responses, sera from the mice immunized with anti-mAbs together with MPLA and CpG as adjuvants were assessed for antibodies against rat IgG. Mice immunized with DNA-TRP-2 plasmid were used as a negative control and their sera did not contain any significant anti-rat IgG compared to mice immunized with 5D3-TRP-2 and the isotype control mAb IgG2a-TRP-2. Relatively lower anti-rat IgG production was induced by IgG2a compared to 5D3-TRP2 in agreement with published results using LPS as adjuvant (McKenzie et al., 2007). Both 5D3 and 6C3 bind to CTLD4-7 domain of MR but 5D3 mAb bind more efficiently as compared to 6C3 while the isotype control does not show any binding. It was originally concluded that differential adaptive immune response in terms of anti-rat IgG production could be due different binding efficiencies of anti-MR mAb (McKenzie et al., 2007). The adaptive immune response obtained in terms of anti-rat IgG could

be due to Ab uptake by APCs through activating Fc receptors which are known to have strong affinity for IgG2a antibodies (Getahun et al., 2004) promoting T-activation and Ag specific Ab production discussed above (Akiyama et al., 2003). Moreover, CpG used as an adjuvant in this study enhances loading of phagocytosed Ag on MHC-II which would activate anti-rat IgG Ab production (Blander and Medzhitov, 2006).

5.4.3 Limitations of the approach and Summary

In conclusion, Immunisation of anti-MR conjugates along with appropriate adjuvants indicated that there is a scope for MR ligands to induce adaptive immune responses, resulting in Ab production and antigen-specific T-cell activation. These findings will benefit the development of novel antigen delivery methods through MR suitable for robust T-cells activation and the development of vaccines against cancer other infectious diseases.

Chemical coupling is an inefficient method of linking Ags with IgG molecules as the degree of conjugation is variable even between IgG molecules conjugated side by side (Sancho et al., 2008). Therefore, anti-MR chimeric antibodies in which Ag was incorporated at the constant region of their heavy chains as described in chapter 3 and 4 were the prefered option. These reagents have several advantages over chemically linked conjugates. Firstly, each IgG molecule contains same amount of Ag. Secondly, the variable region of anti-MR mAb was incorporated into the constant region of mouse IgG1 containing mutations in its Fc region and thereby eliminating the possibility of FcR engagement and lastly, the contstant region chimeric Abs is mouse IgG1, so

the xenogenic responses can be avoided when immunized in mice. On the otherhand, these reagents would not benefit from the potential beneficial effect of engaging MR and FcR simultaneously and the use of a non mutated Fc portion might need to be re-considered.

6 Use of Glycopolymers for Antigen Targeting to dendritic cells potentially through MR

6.1 Introduction

Antigen targeting through different receptors results into different immune outcome (Keler et al., 2007) and even targeting through a single receptor by using antibodies of different affinity induced different immune responses as suggested by the study conducted by Mckenzie et al (McKenzie et al., 2007). Generation of antibodies against different receptors is expensive, difficult, time consuming process. Furthermore it is not easy to generate different antibodies of different affinity against a single receptor. Due to these limitations, a more robust and convenient alternative method was required for antigen delivery to DCs *in vivo*.

In this study glycopolymers have been used to target DC through MR as an alternative to Ab mediated targeting. MR is an endocytic receptor and the binding of ligands is calcium dependent. The CTLD4-7 region of MR has affinity for mannose and mannosylated proteins (East and Isacke, 2002). Antigen targeting through MR leads to cross presentation and the development T-cell immunity which is essential to treat chronic infectious disease and cancer (Burgdorf et al., 2008).

In biology, carbohydrates are involved in a number of crucial events spanning from signal transduction to cellular recognition and can be used as ligands to improve the distribution of drugs and Ag to develop vaccines and other therapeutic agents to treat human diseases (Seeberger and Werz, 2007). Carbohydrates ligands bind to lectins, with C-type lectins being present on the cell surface of APC that can act as receptors with a strong affinity and can be

used to deliver Ag (Lee and Lee, 2000). In normal circumstances, monosaccharides interact weakly with their binding proteins, glycan-binding receptors. To achieve strong interaction for Ag targeting through C-type lectins multivalent interactions are required. These could be achieved by chemical modification of the carbohydrate ligands. Therefore, to get a stronger interaction, carbohydrate molecules are often placed along a polymer backbone and the observed increase in binding efficiency is known as 'the glycocluster effect' (Ting et al., 2010).

Polymers carrying carbohydrate functional groups are known as glycopolymers. Glycopolymers include linear glycopolymers, glycodendrimers and spherical glycopolymers in the form of nanoparticles and vesicles (Pieters, 2009). In 2001, Sharpless and co-workers introduced the concept of "click chemistry", which defines a modular synthetic approach to generate new moieties by joining small units together in order to achieve high yield, as well as generating fewer by-products (Kolb et al., 2001).

Click chemistry has wide applications in the production of glycopolymers for biomedical applications. Potential toxicity due to contamination by residual metal catalysts must be taken into account when traditionally synthesised products are to be used in biological applications (Wang et al., 2003). In copper-catalysed azide-alkyne cycloadditions, a ppm amount of copper remains in the product even after the purification. Therefore, an alternative to such traditional click reactions had to be developed. Chemists are working to develop new "Click" reactions, which can work without the presence of any

metal catalyst and thus avoiding associated metal toxicity to living organisms. Bertozzi first carried out alkyne-azide cycloadditions at room temperature for dynamic *in vivo* imaging without the use of any copper catalyst (Baskin and Bertozzi, 2007, Baskin et al., 2007). Subsequently, in 2008, Lutz presented an alternative idea to perform azide-alkyne cycloaddition without any copper catalyst (Lutz, 2008). Post-polymerisation modifications, curing reactions and certain polymerisation reactions are widely performed by free-radical addition of thiol to a double bond, to facilitae the coupling of peptides with the glycpolymers (David and Kornfield, 2008, Nilsson et al., 2008, Dondoni, 2008, Willcock and O'Reilly, 2010).

This study is a part of a wider project which has the ultimate goal to develop an Ag delivery system and vaccine against cancer. In this study functionalisable ('clickable') polymers were prepared by Atom transfer radical polymerisation (ATRP) starting from an initiator which presented a reactive unit able to react selectively with free cystein protein sulphidryl units. The polymer alkyne repeating units were then clicked with different sugar azides in different relative concentration to generate a library of glycopolymers with different relative amount of sugar units. Binding of different glycopolymers to MR and MR-mediated endocytosis was analysed. In future, glycopolymers will be conjugated with recombinant OVA mini-protein: a protein of molecular weight of 15 kDa and 136 aminoacid residues containing major $CD4^+$ and $CD8^+$ epitopes. Glyco-conjugates will be tested for their potential to induce T-cell-immunity.

6.2 Materials and Methods

6.2.1 Synthesis of glycopolymers

Four different glycopolymers containing 100% mannose, 66% mannose/34% galacose, 34% mannose, 66 % galactose and 100% galactose were synthesised as a part of collaborative project with Dr G. Mantovani (Pharmacy Department, Nottingham University) using click methodology. Oregon green was incorporated into the glycopolymer molecule to facilitate its tracking within the cells as shown in figure 6.1.

6.2.1 Endocytosis assay of glycopolymers by using flowcytometry

Endocytosis assays were performed to test the ability of MR to internalise the different glycopolymers using the protocol as described in section 2.2.5 using mouse bone derived-macrophages, CHO cells, CHO-MR cells and monocyte derived human DC. These cells were generated by following the protocols as given below.

6.2.2 Growing CHO and CHO-MR cells lines

CHO and CHO-MR cell lines were cultured by following the protocol described in section 2.2.6.

6.2.3 Generation of bone marrow derived Macrophages.

Bone marrow was collected form C57Bl/6 mice according to the protocol described in section 2.3.1 and cultured by following protocol described in section 2.3.2. Macrophages were resuspended in RPMI complete media defined in 2.1.2 section and used for endocytosis assays of glycopolymers.

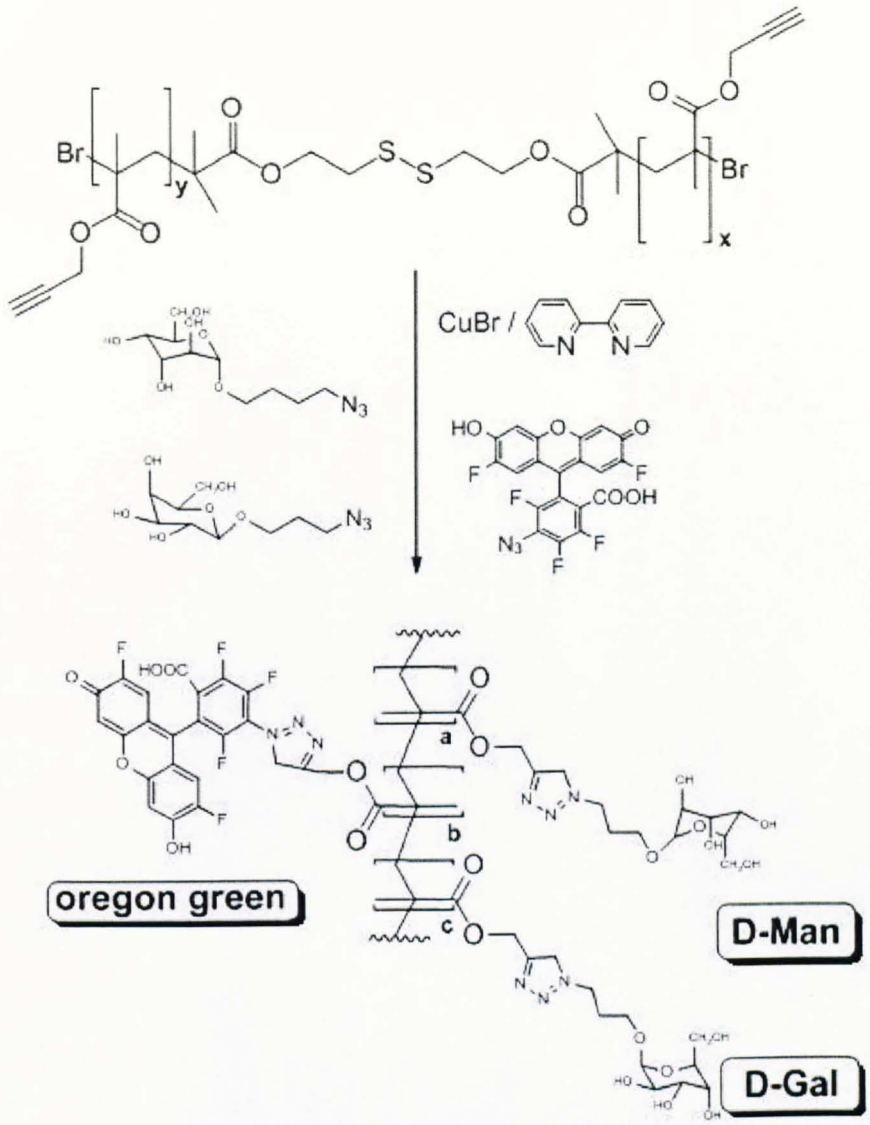


Figure 6.1: Schematic diagram showing the synthesis of glycopolymer.

6.2.4 Generation of monocyte derived human dendritic cells

Monocyte derived human dendritic cells were cultured according to the protocol reported in section 2.3.3 of thesis. Dendritic cells were used at day 6 for the endocytosis assays of glycopolymers.

6.2.5 Analysis of endocytosis by confocal microscopy

Cover slips were sterilised with concentrated sulphuric acid and washed several times with media to remove the traces of acid. 5×10^5 CHO-MR cells were

seeded on the cover slip of each well in 12 well tissue culture plates. Bubbles under the cover slips were removed to stop cells from sticking to the bottom of the cover slip. CHO-MR cells were cultured overnight and washed with PBS followed by the incubation of the cells with different glycopolymers for 5 min, 15 min and 30 min at room temperature. The cells were washed with PBS and incubated with ice-cooled 2% paraformaldehyde hepes buffered saline for 10 minutes at 4°C. Cells were washed again with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI) for 3-5 minutes at room temperature. DAPI is a fluorescent dye which has a strong binding affinity to A-T rich region of DNA. After fixing, the coverslips were mounted onto the slides by using fluorescent mounting media and the coverslips were sealed on the slide by using nail polish on the edges.

6.2.6 Lectin ELISA to analyse the binding of glycopolymers with mannose receptor

Lectin ELISA was performed to analyse the binding of different glycopolymers to different MR-constructs according to the protocol decribed in section 2.2.4.2. Inhibition ELISA was performed to analyse if the binding of glycopolymer to MR is mannose dependent. It was performed as a modification of the technique as described in section 2.2.4.3 in which MR constructs were prepared in TBS-1M NaCl with with glycopolymers ranging from 13mM to 0.13μM.

6.2.7 Generation of the expression vector encoding OVA mini-protein

pBlueRIP vector carrying the full OVA gene was kindly provided by Prof Christian Kurts. It was recovered and transformed into TOP-10 chemically competent *E.coli* and plasmid isolation was done using QIAGEN miniprep kit. Purified plasmids were characterized by restriction digestion with HindIII. Fragment encoding the OVA mini-gene was amplified using a forward primer containing a region complementary to the OVA-minigene, an EcoRI restriction site and a leader peptide (MGWSCIILFLVATATGVHS). A Cystein residue was incorporated at the carboxy terminus of the OVA-mini protein to facilitate its chemical coupling to the glycopolymer. The leader peptide was inserted to assist the extracellular localisation of the OVA-miniprotein. The reverse primer included the NotI restriction site to facilitate cloning and a His-tag to facilitate purification by affinity chromatography as shown in figure using commercially available nickel columns as described in section 2.2.1.2.

The OVA-minigene was PCR amplified by using Phusion enzyme using the following PCR programme:

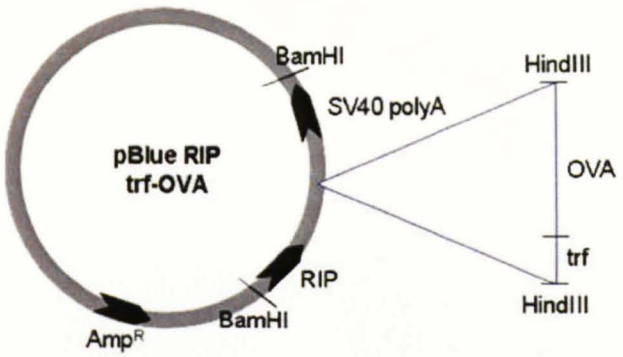
98°C 40 sec. (Denaturation)
45 cycles: 98°C 8sec, 58°C 20 sec, 72°C 30sec.
72°C 5min.

PCR products were analysed by agarose gel electrophoresis (1% w/v) and were extracted from the gel using a Gel extraction kit (QIAGEN) according to manufacturer's instructions. This extracted DNA and the expression vector provided with the DEC205-HEL construct was digested with EcoRI and NotI. Reactions were incubated at 37°C for 2-4 hours. After restriction digestion the

relevant bands were purified by agarose electrophoresis and the DNA was purified. The digested OVA-minigene was ligated into the expression vector with the help of T4 DNA ligase (Roche) using a 1:3 molar ratio insert to vector The ligation mix was incubated at 4°C overnight in a final volume of 10 µl. The ligated plasmids were used to transform One Shot *E.coli* cells (Invitrogen). The transformed cells were incubated overnight at 37°C on LB agar plates containing 100µg/ml ampicillin. Same set of primers used for the amplification of the OVA-minigene were used for the colony PCR screening and using the following PCR programme:

95°C 15 min. (Cell lysis and denaturation)
35 cycles: 94°C 45 sec, 55°C 45 sec, 72°C 1.5 min.
72°C 10 min. (Final amplification)

Positive clones were sequenced at the Biopolymer Synthesis and Analysis Unit, Nottingham University by using the vector specific primers as shown in table 3.4.



Forward Primer
5'-ATTTGAATTCGCCATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAA
CTGCAACTGGAGTACATTCAATGTTGGTGtgcTTGCCTGATGAAG-3'

Reverse primer
5'-AGCTGCGGCCGCTTAGTGATGGTGATGGTGATGTGCAGCATCCACTCCA
GCCTC-3'

Figure 6.2: pBlueRIP carries a full OVA insert of about 2Kb size and was generated by inserting the rat insulin promoter (RIP)/SV40 cassette into multiple cloning site (MCS) (BamHI) of pKs-Blue script vector (Stratagene).

6.2.8 Removal of the N-glycosylation sites from the OVA-minigene by point mutation.

Two N-glycosylation sites NLT and NLS were identified in the OVA-miniprotein and removed by point mutation by designing the special primers using primer3 sftware as shown in table 6.1. Two glycosylation sites were removed in two steps each step involved the PCR amplification of the whole vector carrying the gene of interest and Dpn degradation. The PCR programme used for the point mutation was as follows:

98°C 2 min. (Denaturation)
30 cycles: 98°C 10 sec, 55°C 15 sec, 72°C 6 min.
72°C 10 min. (Final amplification)

For Dpn degradation the PCR product was treated with Dpn enzyme for 4 hour at 37°C to degrade the vector which was used as a template. After Dpn

digestion the PCR amplified vector was transformed into One Shot E.*coli* cells. The transformed cells were incubated overnight at 37°C on LB agar plates containing 100μg/ml ampicilln, 0.1mM IPTG and 40μg/ml X-Gal when white/blue selection was required.

Positive clones were further screened by sequencing to confirm the point mutation. The same procedure was repeated to remove the second glycosylation site except the primers used as shown in table 6.1.

Primer name	*Primer sequence*	*GC content(%)*	*Tm (°C)*
Ponit-1 FP	AATACAACCTCGCATCTGTCTTAATGGCTATGG	42.2	67
Ponit-1 RP	ATTCTGTCTACGCTCCAACATAAAAAGGAGGTA	39.4	66.8
Ponit-2 FP	CAGCCAATCTGGCTGGCATCTCCTC	60	67.9
Ponit-2 RP	CCTCTACGGTCGGTCTAACCGACTT	56	66.3

Table 6.1: Two set of primers designed using primer 3 software in order to remove two N-glycosylation sites from OVA-mini gene by point mutation.

6.2.9 Expression and purification of OVA mini-proteins with and without glycosylation sites

OVA mini-proteins with and without glycosylation sites were purified, analysed using SDS PAGE and Western as described in sections 2.2.1, 2.2.2 and 2.2.3.

6.2.10 Lectin Blot

OVA mini-proteins with and without glycosylation sites were analysed by electrophoresis using 12% Novex Tris-Glycine precast gels from Invitrogen. Tris Glycine running buffer (TGS) was prepared (30.3g Tris, 144g Glycine and

10g SDS made up to 1 L with water) and protein samples were run in XCell *SureLock*™ Mini-Cell from InVitrogen under reducing conditions for 1hr 30mins at 125V. Samples were diluted with 4X sample buffer (0.25M Tris-HCl pH 6.8, 8% SDS, 30% Glycerol, 0.02% Bromophenol Blue and 0.3M DTT) and heated to 95°C for 5 mins prior to loading. Protein concentrations upon loading were 5μg per well; 5μl of a control pre-stained protein ladder (Fermentas) was added to track sample migration in the first lane of the gel.

After gel electrophoresis, nitrocellulose membranes (Amersham, UK) were cut, soaked in Tris Glycine (TG blotting buffer (25mM Tris pH8.5, 0.2M Glycine and 20% Methanol), gel and membrane were assembled in an Invitrogen XCell II™ Blot Module apparatus and soaked with 60 ml of TG blotting buffer. The gels were then transferred for 1 hr at 30V. When finished, the membranes were blocked overnight with 5% BSA in TBS (0.05 M Tris-HCl, 0.15 M NaCl at pH 7.5) at 4°C. In the glycan detection process, the DIG Glycan Differentiation Kit (from Roche Applied Sciences, Germany) was used. The membranes were washed twice with TBS and once with buffer 1 (TBS; 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ at pH 7.5). The required amount of lectin solution was added (for GNA, SNA and DSA 10 μl each; for MAA 50 μl and for PNA 100 μl) to 10 ml of buffer 1. All these lectins that are Digoxigenin (DIG) labelled (Table 6.2) were incubated separately for 1 hr with the membranes and the membranes were then washed three times with TBS to remove any unbound antibodies. The secondary antibody was prepared (10 μl of anti-DIG conjugated to Alkaline phosphatase (diluted in 10 ml of TBS) and the membranes were incubated with the antibody for one hour (Fig 2-1). The detection solution was prepared by using 10 ml Buffer 2 (0.1 M Tris-HCl, 0.05

M MgC_2, 0.1 M NaCl at pH 9.5) diluted in 200 µl NBT/BCIP solution and the membranes were then incubated with the detection solution for 20 mins in the dark (Colorimetric detection). The reaction was stopped by washing with ultra pure H_2O. Membranes were scanned using BioRad GS800 Densitometer. Positive and negative controls are provided with the DIG Glycan detection kit and were used in the western blot experiments.

Labeled lectin	Detection
GNA, Digoxigenin-labeled (Galanthus nivalis agglutinin)	Recognizes terminal mannose, (1-3), (1-6) or (1-2) linked to mannose
SNA, Digoxigenin-labeled (Sambucus nigra agglutinin)	Recognizes sialic acid linked (2-6) to galactose
MAA, Digoxigenin-labeled (Maackia amurensis agglutinin)	Recognizes sialic acid linked (2-3) to galactose
PNA, Digoxigenin-labeled (Peanut agglutinin) PNA, Digoxigenin-labeled	Recognizes the core disaccharide galactose (1-3) N acetylgalactosamine
DSA, Digoxigenin-labeled (Datura stamonium agglutinin)	Recognizes Gal- (1-4)

Table 6.2: The Lectins used to detect glycosylation in allergens. All lectins were purchased from Roche Applied Science, Germany.

The detection of 1,3 fucose was done using anti-1,3 fucose rabbit polyclonal antibody (no. AS07 268, Agrisera, uk). This antibody cross-reacts with fucose residues bound to N-Glycans in alpha 1,3 in plants and insects. The concentration of antibody used was 1µg/10ml of TBS buffer; the reaction mixture was incubated for one hour then washed three times with TBS buffer. Anti-rabbit antibody conjugated with horseradish peroxidise (Sigma) was used as a secondary antibody, again incubated for one hour, washed and the reaction was detected using ECL chemiluminescent detection reagent (GE Healthcare Life Sciences) according to manufacturer's protocol. Membranes were scanned

using BioRad GS800 Densitometer. Bromelain was used as a positive control and Cathepsin from mammalian origin was used as a negative control

6.2.11 MHC-II-restricted antigen presentation of purified OVA mini-proteins to the MF2 T-cell hybridoma

MF2 T-cells were were added into APCs (BMDCs) giving an APC: T-cell ratio 1:1 as described in section 4.2.6.2. Stock solution of commercial OVA (Profos; Hyglos) and prufied OVA mini-proteins were prepared in R10 media and 100µl of serial 2- fold dilution from the stock solution was added per well in duplicates, there by giving a titration of relevant OVA antigen from a top dose of 20µg/ml to 0.62520µg/ml. The amount of IL2 in the supernatants was quantified as described in section 2.2.4.4.

6.2.12 Chemical coupling of the glycopolymers to OVA-mini protein

The cysteine (Cys) residue incorporated into the OVA-mini proteins by genetic manipulation was used to facilitate chemical coupling to glycopolymers through a disulfide bond. Due to the presence of this Cys OVA-mini protein dimerized through disulfide bridges and it was reduced before conjugation. Tris(2-carboxyethyl)phosphine (TCEP), a commercially available reducing agent was used to reduce the OVA-mini protein dimers. 5eq. of TCEP prepared in water was added to 10 mM PBS solution carrying OVA-mini protein and the final solution was stirred at room temperature for 2 hours. 60eq. of the glycopolymers (10 mg) was prepared in 100 mM of PBS (pH 8.0) and mixed with the reduced OVA-miniprotein. The resulting solution was kept at room temperature for few minutes and left to stir overnight at 4°C.

6.2.13 Purification of Glycopolymers conjugates using Dynabeads (Invitrogen).

After the conjugation of the OVA-miniprotein to the glycopolymers the conjugates were purified by using nickel-coated dynabeads. For the purification using dynabeads it is important that the solution should not contain ionic detergents, DTT, DTE, EDTA or any other chelators. TCEP from the conjugation mix was removed by diluting it in water or PBS and concentrating it using an Amicon filter of MWCO 3 KDa. After the removal of TCEP from the conjugation mix the conjugates were purified by using dynabeads according to the manufacturer's instructions.

6.2.13.1 *In vitro* Ag presentation of glycopolymers-OVA conjugates to OVA specific MHC-I and MHC-II T-cell hybridomas.

Antigen presentation of the glycopolymers-OVA conjugates was performed by co-culturing BMDCs and OVA specific T-cells hybridomas. Two types of T-cell hybridomas were used: B3Z, an OVA specific $CD8^+$ T-cell hybridoma, and MF2, a OVA-specific $CD4^+$ T-cell hybridoma.

6.3 Results

6.3.1 Binding of different glycopolymers to MR depends on their D-mannose content.

MR belongs to the class of C-type lectin molecules that binds carbohydrates in a calcium-dependent manner. We used CTLD4-7-Fc and CR-FNII-CTLD1-3-Fc, MR constructs to test the binding ability of the glycopolymers MR by lectin ELISA. Different concentrations (2μg/ml-0.252μg/ml) of the glycopolymers were used according to the standard Lectin ELISA. The binding of the CTLD4-7-Fc MR construct was observed to be dependent on the density of the mannose epitopes present in the glycopolymers and the concentration of the glycopolymers (Figure.6.3-A). There was no binding to the CR-FNII-CTLD1-3 Fc-MR construct by any of the glycopolymers (Figure.6.3 B).

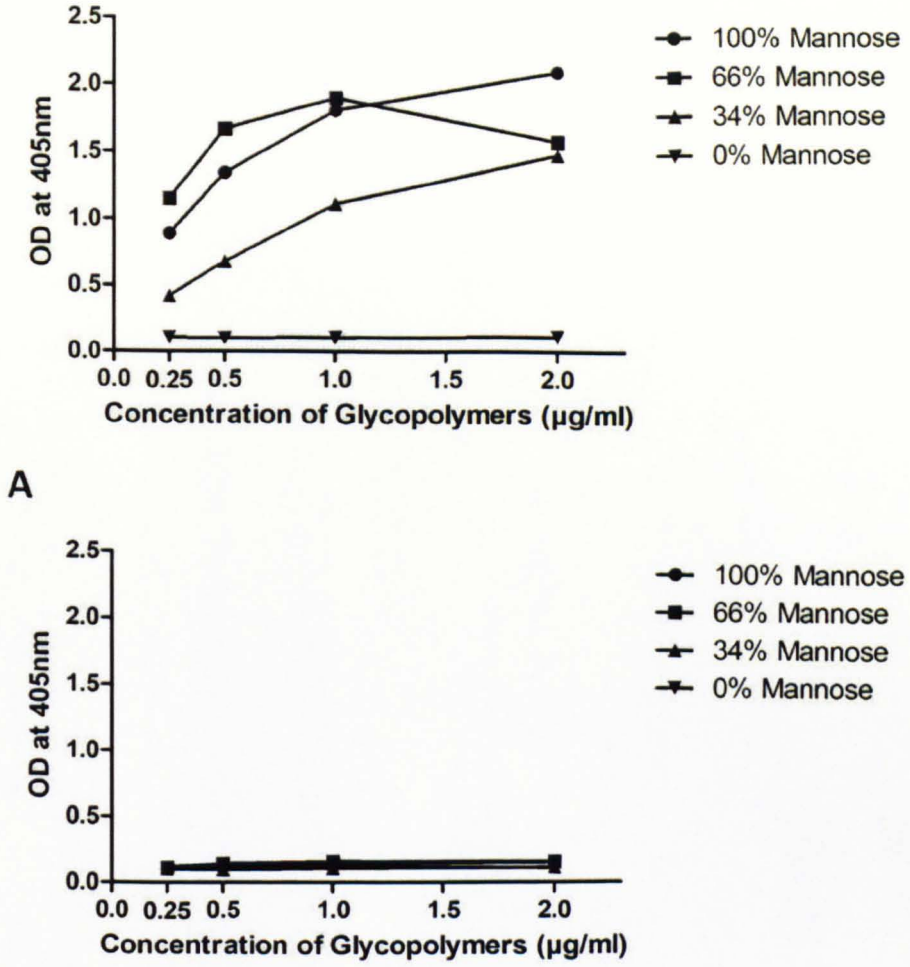


Figure 6.3: Recognition of different glycopolymers containing different percentage of mannose by CTLD4-7-Fc.

Maxi-sorp ELISA plate was coated with glycopolymers containing different percentages of mannose diluted in PBS from 2µg/ml to 0.25µg/ml followed by the addition of 2µg/ml of CTLD4-7Fc (A) or CR-FNII CTLD1-3Fc (B). Anti-human IgG Fc-specific conjugated to AP was used as secondary Ab; the level of binding of different glycopolymer molecules with different MR domains was assessed by measuring OD at 405nm after addition of 1mg/ml of p-nitrophenyl phosphate substrate. Error bar on each column indicates SEM.

Inhibition ELISA was performed to confirm that binding of glycopolymers to CTLD4-7 Fc domain depends on their mannose content. Several dilutions of the glycopolymers (from 13mM to 0.13µM) were prepared and mixed with 2 µg/ml of CTLD4-7-Fc MR. Since mannose has strong binding affinity to

CTLD4-7 Fc domain of MR, the glycopolymers containing more mannose epitopes should show more inhibition of CTLD4-7 Fc binding. Glycopolymer containing 66% mannose showed maximum inhibition at concentration of 0.13mM to 0.13μM. Other glycopolymers inhibited in the following order: 100% mannose > 34% mannose. The glycopolymer containing 0% mannose did not show any inhibition except at higher concentration of 13mM, which was likely due to oversaturation. The optimal level of inhibition of different glycopolymers was seen at the concentration interval 0.13mM to 0.13μM; at higher concentrations complete inhibition was observed due to oversaturation of glycopolymers (Figure 6.4).

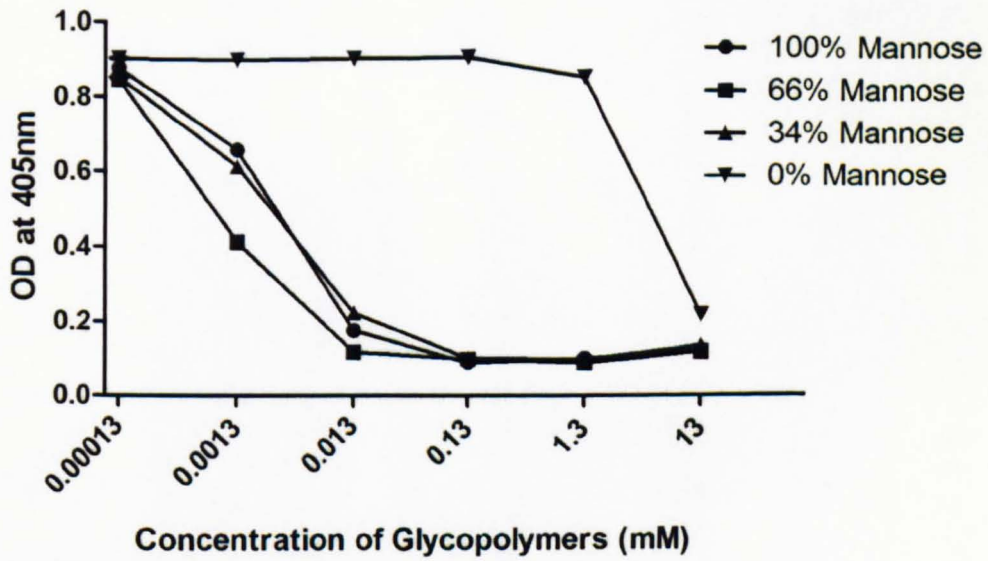


Figure 6.4: Inhibition ELISA to confirm that the binding to the CTLD4-7 region of MR depends on the content of mannose of the glycopolymers.

ELISA plate was coated with mannan. Glycopolymers containing different percentages of mannose were diluted in TBS ranging from 13mM to 0.13μM mixed with 2μg/ml CTLD4-7 Fc MR. Anti-human IgG Fc-specific conjugated with AP was used as secondary antibody and the level of inhibition of CTLD4-7Fc by different glycopolymers was assessed by taking OD at 405nm after addition of 1mg/ml p-nitrophenyl phosphate substrate. Error bar on each column indicates SEM.

6.3.2 Glycopolymers containing mannose are internalized by MR-in differenT-cells.

Glycopolymers containing terminal mannose binds to MR as shown in the lectin ELISA. The endocytosis potential of glycopolymers was assessed by endocytosis assay using monocyte-derived human DCs (moDCs) and bone marrow-derived macrophages (BM-macrophages). Both types of cells express MR as shown in figure 6.5. The level of endocytosis of different glycopolymers was assessed using flow cytometry and fluorescent microscopy by tracing the Oregon green fluorescent dye. Glycopolymers containing 100%, 66% and 34% mannose showed similar endocytosis in moDCs, but in BM-macrophages endocytosis levels were as follows: 100% mannose=66% mannose>34% mannose. Additionally, glycopolymers containing 0% mannose was not endocytosised, which indicates that the endocytosis of the glycopolymers is mediated by a mannose-binding lectin. Endocytosis of the glycopolymers is dose-dependent as they were better endocytosed at concentrations of 10 μg/ml relative to 5μg/ml in the case of moDCs, while in BM-macrophages, a gradual decline in endocytosis from highest dose of 10μg/ml to 0.625μg/ml was observed. After endocytosis for different time spans, CHO-MR cells were also analysed by confocal microscopy and similar endocytosis levels was observed in glycopolymers containing 100%, 66% and 34% mannose; glycopolymers containing 0% mannose showed relatively less endocytosis (Data not shown).

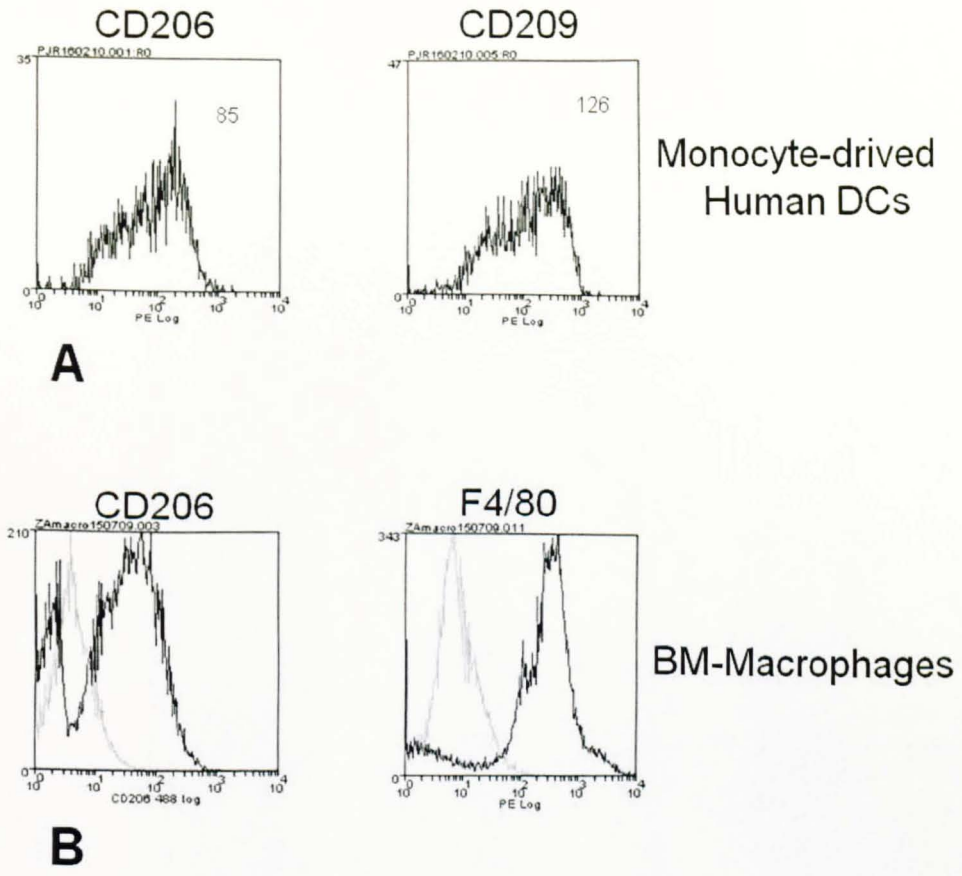


Figure 6.5: Confirmation of MR (CD206) expression on monocyte derived DCs and BM-macrophages by flow cytometry.

Monocyte-derived human DCs (A) and macrophages derived from mice bone-marrow (B) were cultured and expression of CD206 (MR), CD209 and F4/80 was analysed by FACS.

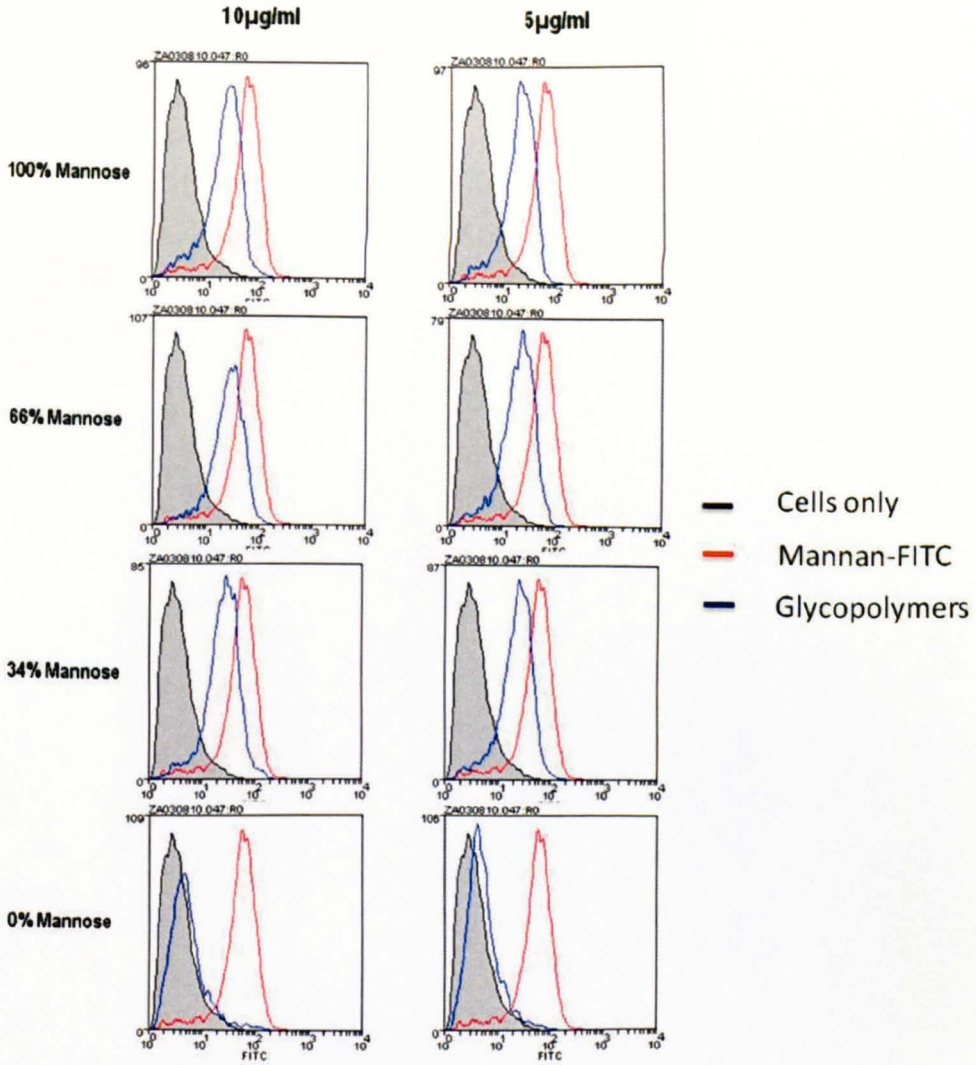


Figure 6.6: Flowcytometric analysis to assess endocytosis of different glycopolymers by human moDCs.

Monocyte-derived human DCs were cultured and on day 6 DCs were incubated (30 minutes) with glycopolymers containing 100% mannose, 66% mannose, 34% mannose and 0% mannose labelled with oregon green. After incubation cells were washed with PBS, fixed with 2% formaldehyde and endocytosis was assessed by flow cytometry.

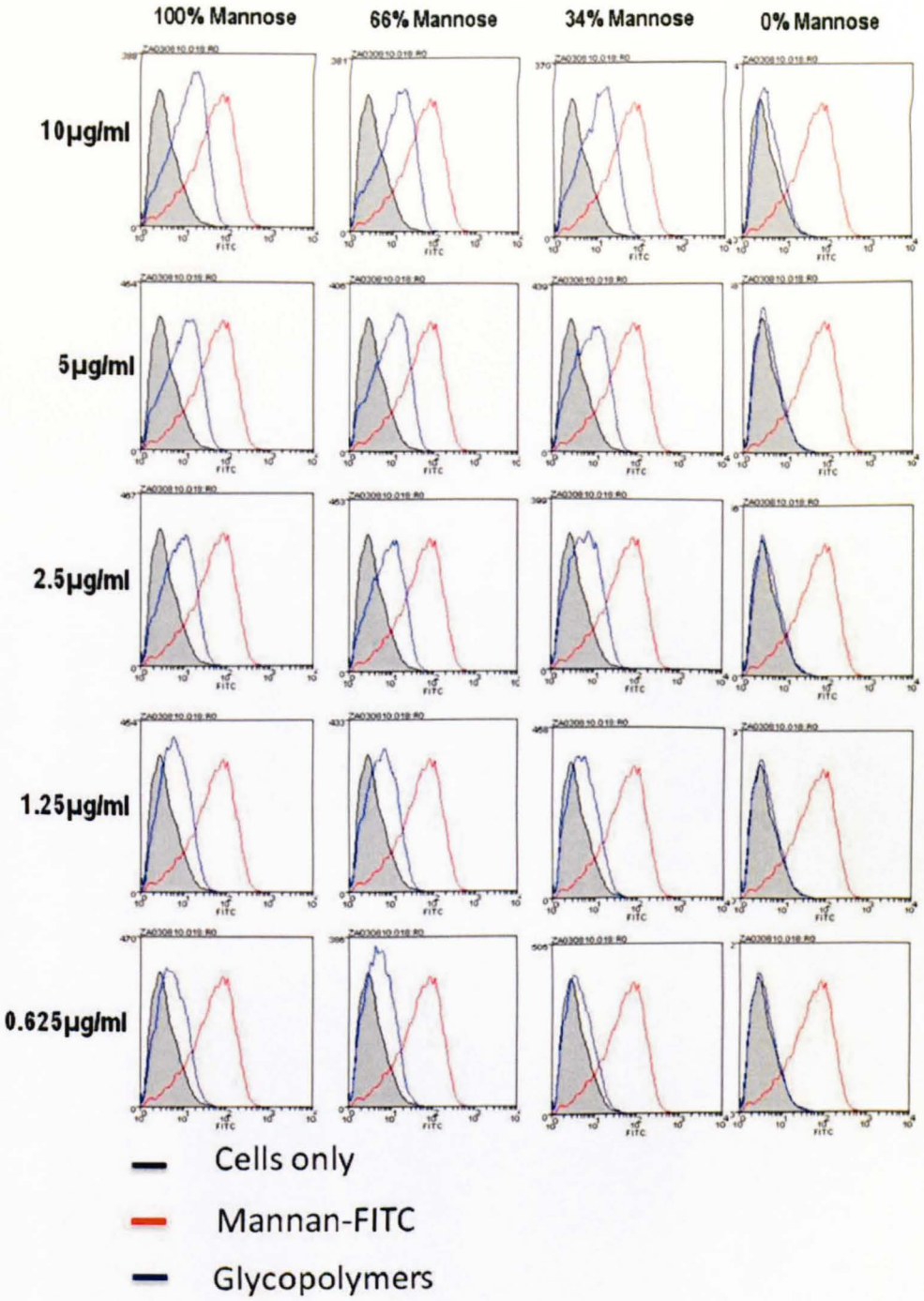
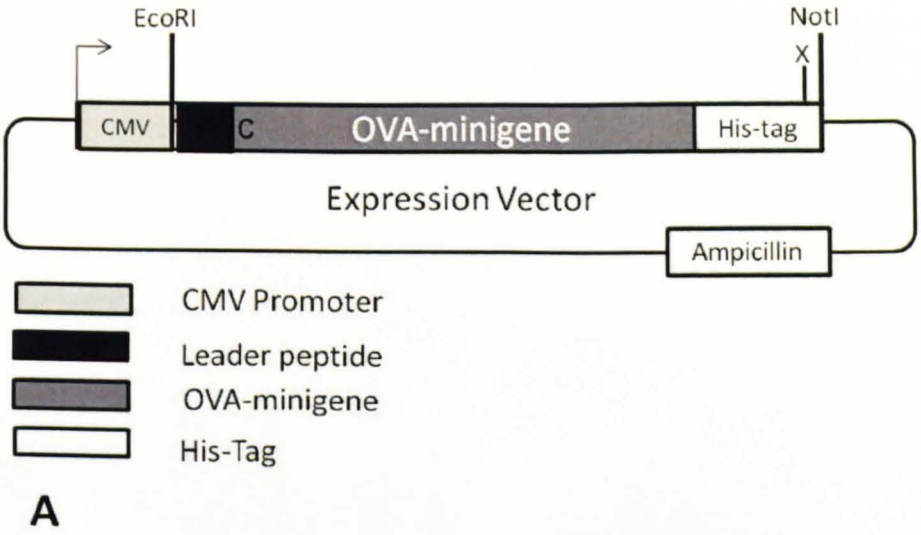


Figure 6.7: Flowcytometric analysis of the endocytosis of different glycopolymers by bone marrow derived macrophages.

Macrophages derived from mice bone-marrow were incubated for 30 minutes with glycopolymers containing 100% mannose, 66% mannose, 34% mannose and 0% mannose at concentrations ranging from 10µg/ml to 0.625µg/ml. After incubation cells were washed with PBS, fixed with 2% formaldehyde and endocytosis was assessed by flow cytometry.

6.3.3 Synthesis of recombinant OVA-mini proteins

OVA-minigene was subcloned from full OVA gene using specific primers. The primers were designed in such a way that a leader peptide followed by cysteine residue was incorporated at 5'end and a His-tag was added at the 3' end of the OVA-minigene to facilitate protein purification (Figure 6.8A). OVA-mini protein was cloned into an expression vector and positive clones were screened by colony PCR and DNA sequencing. In order to generate an aglycosylated OVA-mini-protein, its two N-glycosylation sites were removed by point mutation using two sets of primers as described in Materials and Methods (Figure 6.8B). After removal of glycosylation sites the positive clones were screened by colony PCR and DNA sequencing. Both glycosylated and aglycosylated OVA-miniproteins were expressed in HEK-293T and purified by affinity chromatography as described in section 2.2.1.2. The purified OVA-miniproteins were concentrated using amicon ultra column (Millipore) following the manufacturer instructions. The concentrated OVA-mini proteins were quantified by BCA protein quantification method and analysed by western blotting using polyclonal anti-OVA antibody as shown in figure 6.9. Western blotting confirmed that the protein purified is the OVA-miniprotein.



A

MGWSCIILFLVATATGVHSMLVCLPDEVSGLEQLESIINFEKLTEWTSS
NVMEERKIKVYLPRMKMEEKYNLTSVLMAMGITDVFSSSANLSGISS
AESLKISQAVHAAHAEINEAGREVVGSAEAGVDAAHHHHHHstop-
codon.

B

Figure 6.8. Schematic representation the expression vector for the OVA-minigene (A) and its protein sequence (B).

Vector carrying OVA minigene has a CMV promoter and EcoRI restriction site at the 5' end and His-tag and NotI restriction site at 3' end (A). Protein sequence of OVA-miniprotein with leader sequence at the N terminus shown in blue text, OVA sequence in black and His-tag at C-terminus shown in blue text. The OVA-minigene has two N-glycosylation sites NLT and NLS shown in red text which were removed by point mutation (B).

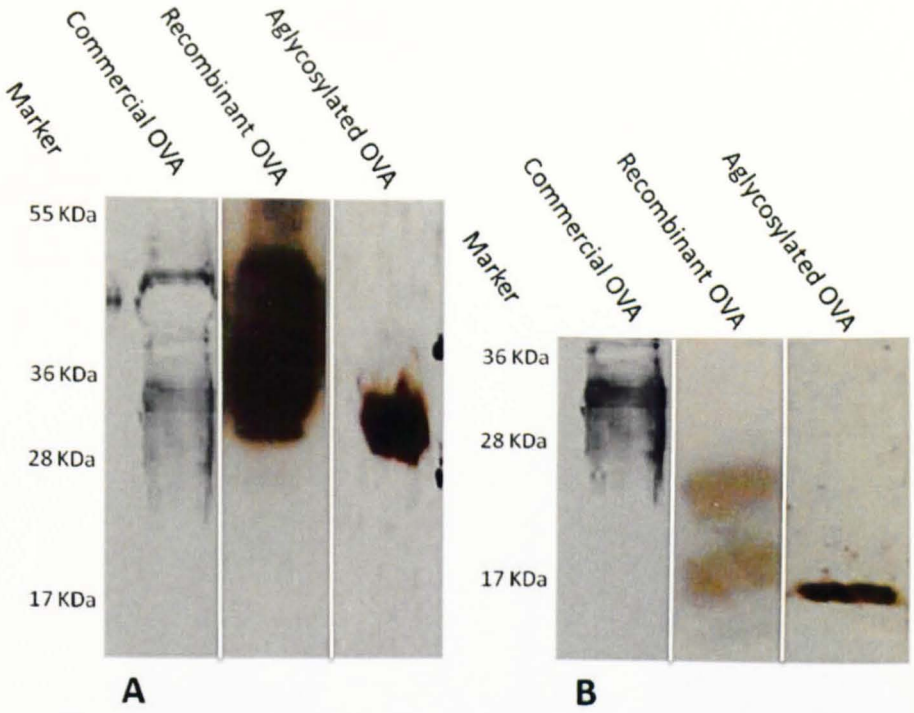


Figure 6.9: Western Blot analysis using polyclonal anti-OVA antibody to confirm the identity of purified OVA-mini proteins.

200ng of commercial OVA, recombinant OVA-mini and aglycosylated OVA-mini were run in a 12% polyacrylamide gel under non-reducing (A) and reducing conditions (B). The gel was transferred to nitrocellulose membrane and blocked in blocking buffer. OVA was identified using a rabbit polyclonal anti-OVA antibody and HRP-goat anti-rabbit antibody.

6.3.4 Recombinant OVA-mini proteins do not bind to MR due to the lack of mannosylation.

Binding of both glycosylated and aglycosylated OVA-miniproteins to the MR domain CTLD4-7 was assessed by lectin ELISA. Commercial OVA showed binding (in agreement with data shown in Chapter 3) but OVA-miniproteins did not as shown in figure 6.10. Binding of MR to proteins depends on their level of mannosylation. The glycosylation pattern of commercial OVA and recombinant OVA-miniproteins was assessed by lectin blot with the help of Dr. Abeer Al-Ghouleh and Dr. Ameer Ghaemmaghammi, by following the protocol as described in section 6.2.11. Commercial OVA and recombinant

OVA-miniprotein are highly glycosylated with terminal galactose as compared to aglycosylated OVA-miniprotein. Commercial OVA also showed terminal mannosylation, however neither glycosylated nor aglycosylated OVA-miniproteins showed mannosylation. None of the OVA proteins showed terminal sialic acid as shown in figure 6.11. Glycosylation analysis proved that recombinant OVA-miniproteins did not show binding to MR probably due to the lack of mannosylation, while commercial OVA binds to MR due to its terminal mannosylation.

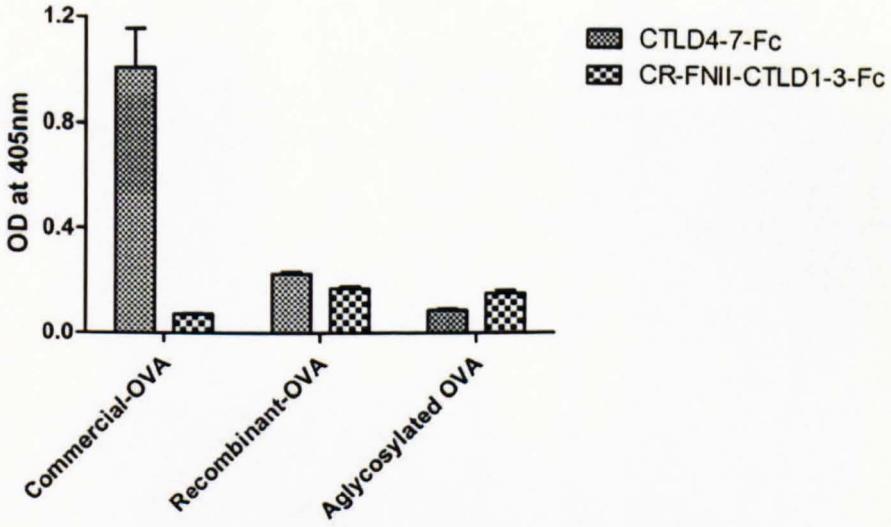


Figure 6.10: Binding analysis of commercial OVA and purified OVA-mini proteins to CTLD4-7-Fc.

ELISA plate was coated with 5μg/ml of commercial OVA and purified OVA-mini proteins and incubated overnight at 4°C, followed by the addition of 5μg/ml of CTLD4-7-Fc and CR-FNII-CTLD1-3- Fc. Anti-human IgG Fc specific conjugated with AP was used as secondary antibody; the level of binding of different OVA molecules with different MR domains was assessed by taking OD at 405nm after addition of 1mg/ml of p-nitrophenyl phosphate substrate. Error bar on each column indicates SEM.

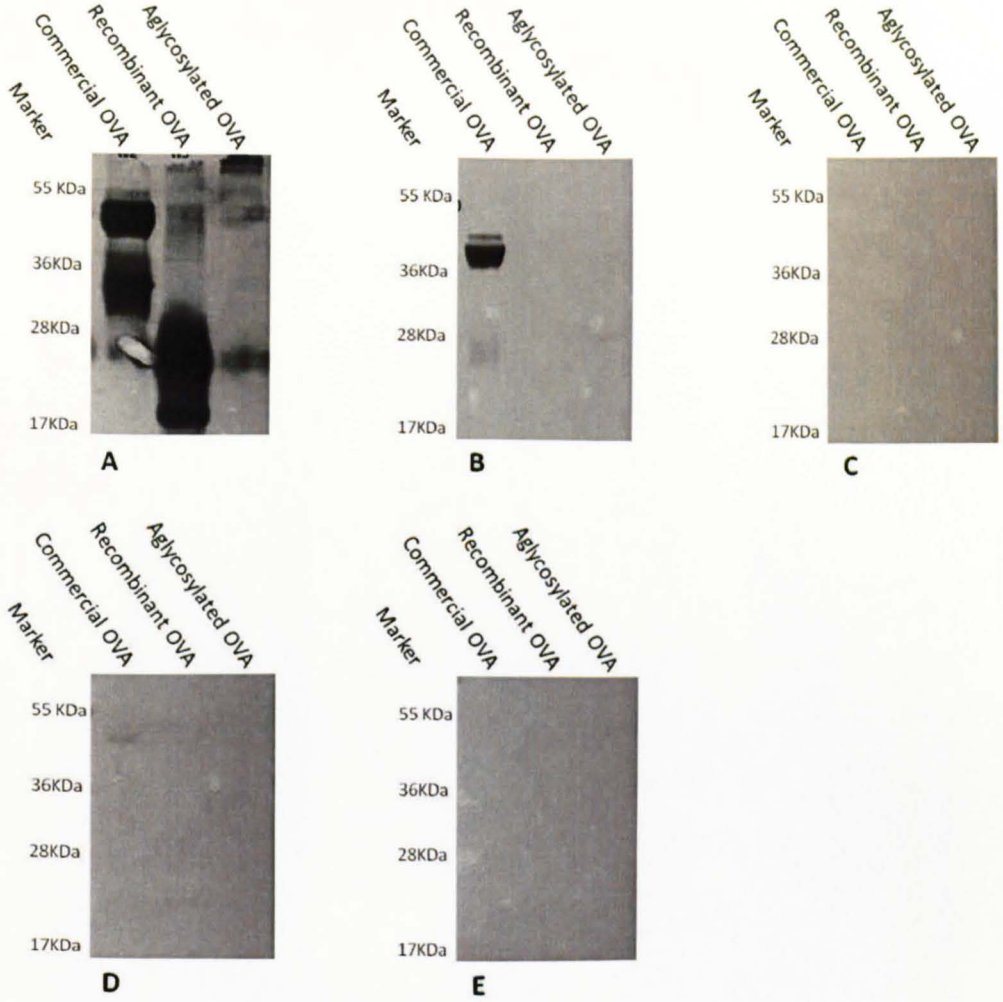


Figure 6.11: Terminal carbohydrate analysis of commercial OVA and purified OVA-mini proteins using DIG glycan differentiation kit.

Different OVA proteins (3μg) were separated on 12% SDS polyacrylamide gel and transferred to nitrocellulose membrane. After immobilisation the membranes were blocked, followed by one hour incubation with different lectins labelled with Digoxigenin: (A) DSA recognises terminal galactose (B) GNA recognises terminal mannose (C) MPA recognises sialic acid (D) PNA recognises terminal galactose (E) SNA recognises sialic acid. The presence of different terminal sugar units was identified by anti-Digoxigenin-AP and staining solution provided with the kit (Roche Applied Sciences, Germany).

6.3.5 DC pulsed with recombinant OVA-miniprotein are more efficient activators of T-cell proliferation than commercial OVA.

T-cell activation potential of commercial OVA and recombinant OVA-miniproteins were compared by culturing BMDCs (both (WT and MR-/-) together with the OVA-specific $CD4^+$ T-cell hybridoma MF2 in the presence of different OVA and the amount of IL-2 in the supernatant was measured. Aglycosylated OVA-miniprotein was found less effective in inducing T-cell proliferation compared to recombinant-glycosylated OVA-miniprotein in both MR+/+ and MR-/- DCs. The degree of T-cell proliferation was the same when DCs were fed with 20 μg/ml of recombinant OVA-miniprotein and 100μg/ml of commercial OVA. Apparently, glycosylated OVA-miniprotein appeared to be 5-fold more potent in inducing T-cell proliferation relative to commercial OVA as shown in figure 6.12. Since for the same amount of protein there will be more molecules of miniOVA than of commercial OVA, furher work is required to determine if mini-OVA could be better presented than unmodified OVA. The robust T-cell proliferation in response to OVA induced by MR-/-DC was surprising as MR has been shown to be involved in OVA uptake. Further dilutions of the Ag are required to address this issue. T-cell proliferation as assessed by IL-2 measurement was relatively low with OVA-miniprotein having no N-glycosylation sites, which was further reduced in MR-/- DCs (Figure 6.12B).

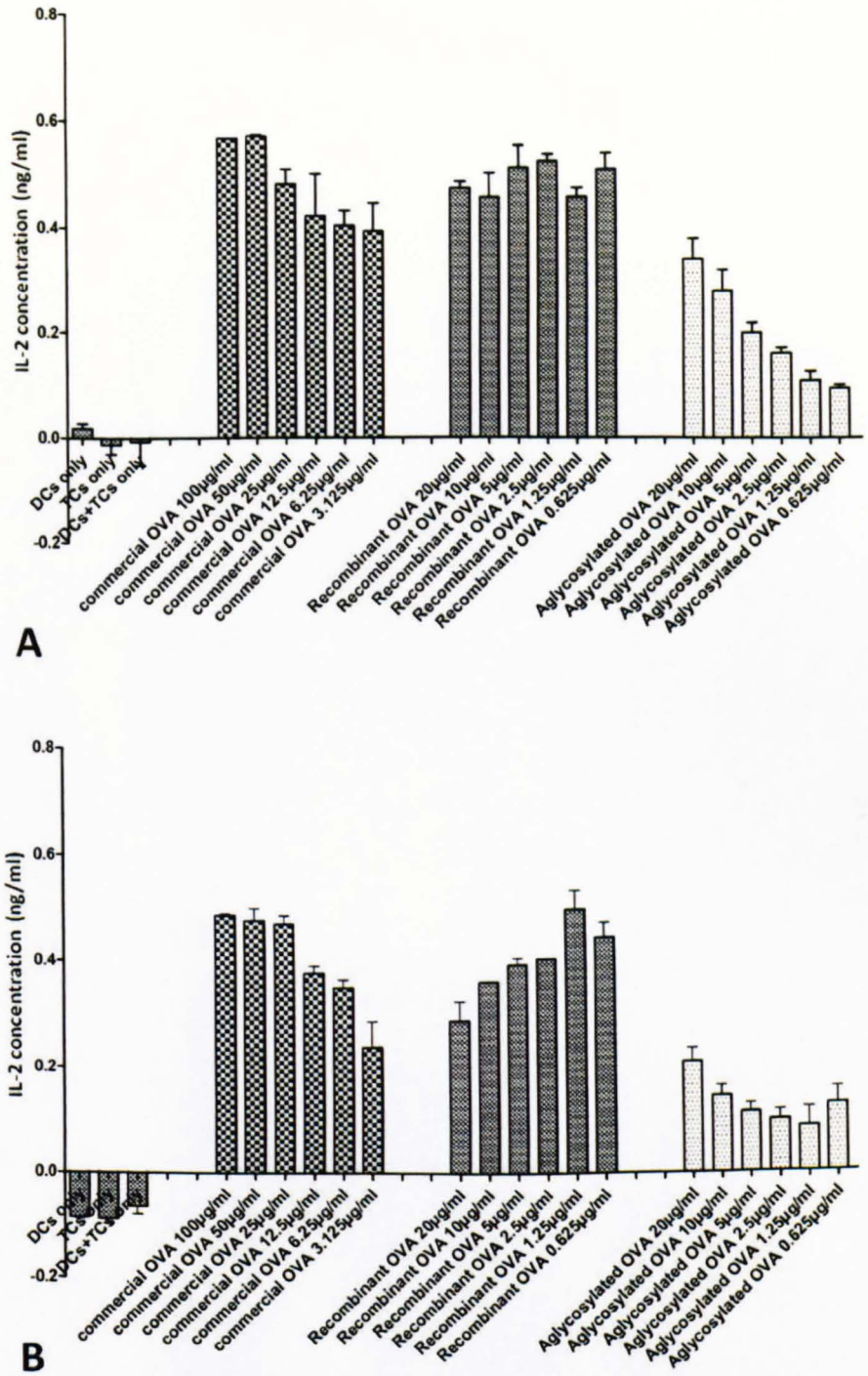


Figure 6.12: $CD4^+$ T-cell prolifaertion in response to commercial OVA, recombinant OVA and aglycosylated OVA protein using co-cultures of BMDC and MF2 T-cell hybridoma.

DCs (5×10^4 cells) obtained from the bone marrows of MR+/+ (A) and MR-/- (B) mice were fed with different OVA proteins at a range of concentrations from 100µg/ml to 0.625µg/ml, and co-cultured with 5×10^4 MF2 T-cells overnight at 37°C and 5% CO_2. The level of OVA-specific T-cell proliferation was estimated by measuring the IL-2 cytokine in the supernatant of the co-culture. The error bar on each column indicates SEM (n=1).

6.4 Discussion

The aim of this study was to assess how antigen targeting using novel glycopolymers could affect the processing and presentation of the model antigen OVA. For this we tested their binding to MR a major C-type lectin receptor expressed by DCs.

Four different types of glycopolymers containing 0%, 34%, 66% and 100% mannose were generated by click chemistry and a thiol (SH) group was incorporated into the polymer to facilitate efficient conjugation of these glycopolymers to model antigens through a disulfide bond. Similarly a fluorescent dye, Oregon green was included into the backbone of the glycopolymers in order to track these molecules inside the cells. Glycopolymers were tested for MR-binding and their ability to bind MR was found to be dependent on the amount of mannose monomers on the glycopolymer backbone. The glycopolymer containing 0% mannose and 100% galactose did not show any binding to MR. This is in agreement with other studies in which it has been reported that the amount of mannose in the polymer linearly correlated to their binding to ConA, a lectin specific for binding to α-D-mannosyl and α-D-glucosyl residues in terminal position (Cairo et al., 2002, Gestwicki et al., 2002). Endocytosis of the glycopolymers was investigated using monocyte-derived human DCs and bone-marrow derived macrophages expressing MR as shown in figure 6.6 and 6.7. The endocytosis was also found to be dependent on the presence of mannose monomers and MR is capable of internalising mannose-containing glycopolymers. However, glycopolymers containing 100% mannose and 66% mannose showed the same

level of binding to MR and endocytosis probably due to saturation or high density of mannose monomers on these glycopolymers. Interestingly the glycopolymers containing 345% mannose showed reduced MR binding in lectin ELISA but was endocytosed as efficiently as glycopolymers containing 100% and 66% mannose, indicating that poor MR-binding does not have to translate into poor internalisation. Glycopolymers have multivalent interaction with lectin receptor and during this interaction a significant amount of the sugar monomers is not involved in the binding process due to their high density on the glycopolymer backbone. In order to achieve good binding the distance between the two sugar monomer on the glycopolymer should be the same as distance between the two receptors, therefore flexible polymers with moderate sugar density show better binding (Teruaki Hasegawa, 1999).

Different approaches used for antigen targeting through MR to induce immunity involves Ab-mediated targeting (He et al., 2007) or using MR ligands as described in section 1.7. Ligand mediated Ag targeting has been successful, for example oxidized mannan linked with the MUC1 antigen (highly expressed by adenocarcinoma) can target DCs through MR. It effectively induces MUC1 specific CTL immunity (Apostolopoulos et al., 2000); during phase I clinical trials of patients with advanced carcinoma, oxidized mannan linked with the MUC1 antigen induced tumour specific humoral responses in 50% of the patients, but did not successfully induce tumour specifc CTL immunity (Karanikas et al., 1997). However, in phase III clinical trials, patients with stage II breast cancer oxidized mannan-MUC1

conjugates showed promising results, and the patients receiving this immunotherapy were free of tumour recurrence (Apostolopoulos et al., 2006).

Glycopolymers bind are a better choice for antigen delivery and vaccine development as compared to other methods mentioned above as they can be easily manipulated to change the content of mannose residue which directly affects their binding properties to lecting receptors. Therefore, libraries of glycopolymers having different binding capacities can be generated and tested for their potential to deliver Ag and trigger T-cell activation. It has been established that glycopolymers or carbohydrates, having high off rate or dissociation rate are more potent for Ag delivery and cross presentation which is a critical mechanism involved in the development of vaccines against cancer and other infections which need CTL based immunity (Howland and Wittrup, 2008). Synthesis of the glycopolymers employs simple click chemistry and is relatively easy and less inexpensive to synthesise glycopolymers on a large scale. Recently, Singh et al. reported that OVA linked to glycans such as 3-sulfo-Lewis^A or N-acetylglucosamine targets DCs specifically through MR and enhance its cross presentation. Therefore, to avoid binding of glycopolymers to multiple receptors the glycopolymers containing 3-sulfo-Lewis^A or N-acetylglucosamine monomers could be prepared to target antigen specifically through MR.

Commercial OVA is highly glycosylated with terminal mannose and binds MR; it has thus been used as model antigen in MR-related antigen targeting studies (Burgdorf et al., 2007, Burgdorf et al., 2008, Burgdorf et al., 2010,

Burgdorf et al., 2006). In our study, we generated a shorter form of OVA (OVA-mini) containing MHC-I and MHC-II epitopes by genetic engineering as shown in figure 6.8. A leader peptide and a cysteine residue were added at the 5'end and a His-tag was added at 3'end. The leader peptide and His-tag were inserted to facilitate protein secretion and its purification while the cysteine residue will facilitate coupling to the glycopolymers designed for lectin-mediated targeting. N-glycosylation sites in the OVA-miniproteins were removed by point mutation to ensure that binding of the glycopolymer-OVA conjugates was not through OVA. After the generation of the OVA-miniproteins their integrity was confirmed by western blotting using polyclonal anti-OVA Ab (Figure 6.9). The OVA mini-proteins (14KDa) contain cysteine and form dimers (28KDa) through a disulfide bridge as seen by western blot. Additionally, lectin ELISA showed that commercial OVA binds to MR, unlike the OVA-miniproteins generated in our lab (Figure 6.10). In order to confirm that binding of commercial OVA to MR depends on its mannosylation, glycosylation of the commercial OVA and OVA-miniproteins was assessed by lectin blot. Only commercial OVA showed terminal mannosylation which correlated with its ability to bind MR. The OVA-mini proteins did not show terminal mannosylation however, they contain terminal galactose.

Presentation of different OVA molecules to OVA-specific MHC-II restricted T-cell hybridoma (MF2) was assessed using co-culture of BMDCs and MF2, the T-cell hybridoma. The glycosylated OVA-mini protein appeared to induce $CD4^+$ T-cell proliferation more effectively, while aglycosylated OVA miniprotein induced T-cell proliferation to a lesser extent. As indicated in the

lectin blot, the OVA mini-proteins contains only terminal galactose which was absent in the aglycosylated OVA-mini-protein. Macrophage galactose binding lectin (MGL) is expressed by macrophages and recognizes galactose or N-acetylgalactosamine (Kawasaki et al., 1986, Higashi et al., 2002). Sandar et al. reported that MGL-mediated uptake of soluble Ag results in efficient Ag presentaion to $CD4^+$ T-cells. Therefore, the OVA-mini proteins could be targetd through MGL and higher $CD4^+$ T-cell proliferation obtained by glycosylated OVA-mini could be due to its high terminal galactose content as compared to aglycosylated OVA-mini. Overall, the $CD4^+$ T-cells proliferation was found to be independent from MR as T-cell proliferation was not affected by using BMDC derived from MR-/- mice, which is in agreement to the studies reported previously (Burgdorf et al., 2007). Ag presentation is affected by the rate of internalisation, cellular trafficking and processing rate. Additional work investigating the rate of uptake of all OVA preparations using equal number of molecules needs to be done. It needs to be considered that commercial OVA contains different glycoforms that will be internalised probably through different mechanisms (Burgdorf et al., 2010).

Conjugation of the OVA-mini protein to the one of the glycopolymer was attempted by following the procedure described in section 6.2.13. OVA-mini protein has the property to form dimers due to the presence of a cysteine, included to facilitate the formation of disulfide bonds. In the first step of conjugation, an excess of TECP was used to break the disulphide bond between the monomers. A thiol group was inserted into the glycopolymers and

they were coupled to the OVA-mini by a disulfide bridge as shown in figure 6.13 (Jones et al., 2009).

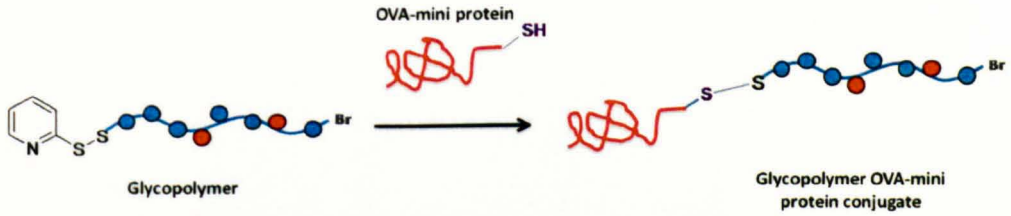


Figure 6.13: General scheme for the conjugation of glycopolymers and OVA-mini.

SDS-PAGE and Western blotting was performed to analyse the conjugation of OVA-mini protein to the glycopolymer. SDS-PAGE results showed the formation of a smear with molecular weight between 72 KDa to 250 KDa indicates the formation of conjugates as shown in the figure 6.14. But the conjugation was not efficient (Figure 6.14) probably due to incomplete reduction of the OVA-mini protein which makes the cysteine residue unavaiable for the conjugation. Further optimization of the conjugation reaction is required in order to reduce dimers of OVA-mini by increasing the quantity of TCEP in the conjugation reaction or an alternate protocol for protein conjugation and monitoring could be followed as reported by Dirks et al. (Dirks et al., 2009).

In the future the different glycopolymers synthesised and characterised in this study will be coupled to recombinant OVA-miniproteins, and their potential to target DC both *in vitro* and *in vivo* to induce OVA-specific $CD4^+$ and $CD8^+$ T-cell proliferation will be assessed. If glycopolymers-OVA conjugates demonstrate improved T-cell activation then they could be used to target the

melanoma antigen TRP-2 to assess their potential to activate TRP-2-specific IFN-γ secreting cells by ELISPOT assay and protect against tumours.

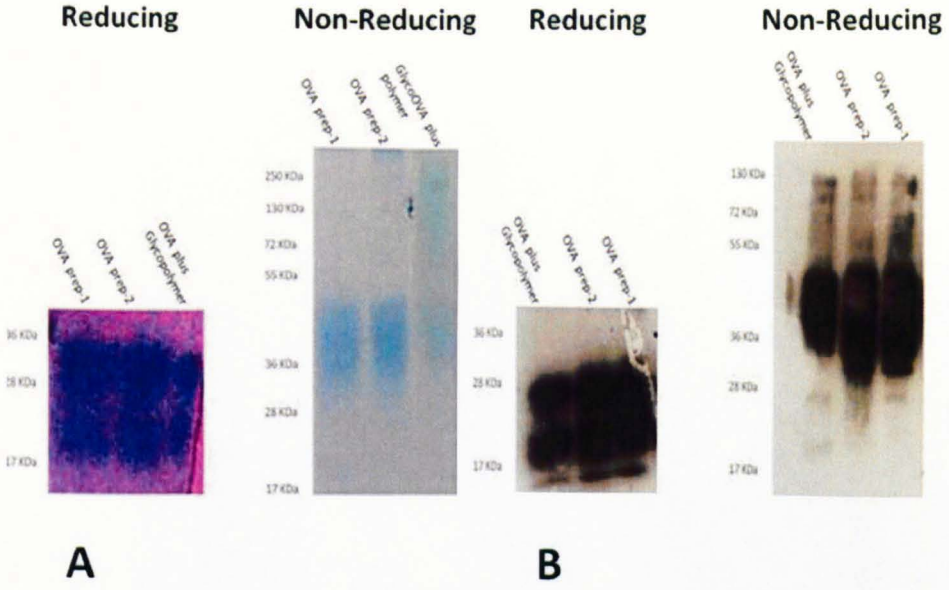


Figure 6.14: Chemical conjugation of glycopolymers with OVA-mini protein and their analysis by (a) SDS-PAGE and (b) Western blotting.

(A) OVA-mini protein only and in conjugated form were separated in 12% acrylamide gels under reducing and non-reducing conditions, respectively, and stained with Simply Blue safe stain. (B) OVA-mini protein in conjugated and unconjugated form was loaded as sample on 12% SDS-PAGE under reducing and non reducing conditions and detected by using Rabbit anti-OVA polyclonal antibody.

In summary, a library of fluorescent glycopolymers with different binding ability to MR was synthesised effectively by using ATRP and click chemistry. Attempts were made to conjugate OVA-mini protein to the glycopolymer but this needs further optimization. Glycopolymers have a natural affinity to CLRs, and more specifically to MR expressed on DCs; thus they have a lot of scope in the development of vaccines by promoting Ag delivery to DCs.

7 General discussion and future directions

7.1 Introduction

A drastic development in the field of vaccine technology has occurred due to the better understanding of pathogenic antigens and the processes leading to the induction of immunity. These findings have paved the way for the rational design and production of safe and more effective vaccines against emerging infectious agents, tumours and autoimmune diseases.

DCs have been used directly as an adjuvant in immunotherapy (Mohamadzadeh and Luftig, 2004) but with limited success as the methodology is very expensive, complex and time consuming. Additionally, antigen-loaded DCs accumulate at the site of injection and only 1-2% of the antigen pulsed DCs reach the local lymphoid organ. Due to these limitations alternative approaches have been developed to target DCs directly *in vivo* in the presence or absence of infectious signals (Shortman et al., 2009, Tacken et al., 2006). Selective Ag targeting to DCs through a specific pathway is critical for the development of vaccines. MR is an endocytic receptor; type-I protein of molecular weight 175 KDa that was the first discovered member of the MR-family. It was found to be involved in the clearance of endogenous glycoproteins. Targeting of antigen through MR using anti-MR antibody (Ramakrishna et al., 2004, He et al., 2007, Tsuji et al., 2010) or glycosylated Ags such as the model antigen ovalbumin (OVA) results in cross presentation and activation of cytotoxic T-cell responses. These fascinating finding makes MR a strong candidate for the development of therapeutic vaccines against viral infections and tumours.

The aim of this study was to develop tools to deliver Ag through MR and investigate how the interaction between MR and a MR ligand-Ag influences Ag processing and T-cell differentiation. Three different novel reagents were generated to verify the hypothesis. (i) Anti-MR mAbs: 5D3 and 6C3 chemically linked to the melanoma epitope TRP-2 by a covalent bond; (ii) MR-specific chimeric Abs fused to HEL and OVA Ags and (iii) novel glycopolymers having different mannose content that will be linked to OVA-mini and tested *in vitro* and *in vivo*.

7.2 Antibody-mediated Ag targeting through MR

Targeting Ag through antibodies against DC receptors is the ideal technique for Ag delivery to DCs due to their specificity and higher affinity (Keler et al., 2007). Receptors used in Ag targeting mostly belong to the C-type lectin receptor (CLR) family, which are calcium-dependent lectins and share primary structural homology in their carbohydrate recognition domain (CRD). The common CLRs used in targeting studies are the mannose receptor (MR, CD206) (Ramakrishna et al., 2004, He et al., 2007, Tsuji et al., 2010), DEC-205 (Hawiger et al., 2001, Bonifaz et al., 2002, Bonifaz et al., 2004, Boscardin et al., 2006), and the DC-specific intercellular adhesion molecule-3 (ICAM3)-grabbing non-integrins (DC-SIGN) (Tacken et al., 2006). Ag targeting through different receptors might result in different immune outcomes due to different intracellular receptor routing, signalling pathways and expression patterns (Tacken et al., 2007). Ramakrishna et al. first described MR targeting by using human anti-MR mAb (B11) fused to the melanoma Ag, pmel17 (B11-pmel17) that lead to Ag presentation in the context of MHC-I and MHC-II molecules, resulting in antigen specific $CD4^+$ and $CD8^+$ T-cell proliferation *in vitro*

(Ramakrishna et al., 2004). In order to perform *in vivo* studies transgenic mice expressing hMR were generated and immunized with B11-OVA along with CpG that induced OVA specific tumour immunity, while WT mice failed to induce protective immunity (He et al., 2007). Human anti-MR Ab was employed to target Ag in the studies mentioned above; it had a xenogenic response when immunized into the mice. We generated mouse anti-MR chimeric Abs to be tested in the mice in order to avoid the xenogenic effect against the chimeric Abs.

The study was started with the aim to generate anti-MR conjugates by chemical coupling of Ag to anti-MR mAb. In this study, MR specific mAb (5D3 and 6C3), and isotype control IgG2a (Martinez-Pomares et al., 2003) were conjugated covalently to TRP-2 and MHC-I restricted melanoma Ag as described in chapter 5. As previously established, MR is expressed by murine bone marrow-derived DCs and other subtypes of DC present in mice (Linehan et al., 2001, McKenzie et al., 2007). This study was performed to assess the potential of anti-MR-TRP-2 conjugates to target DC *in vivo* and activate $CD8^+$ T-cells. Binding of antibodies to MR before and after conjugation and other *in vitro* assays such as coupling efficiency of TRP-2 to IgG molecules, MR-mediated endocytosis and Ag presentation assay were performed before *in vivo* studies.

Initial immunisations performed using MPLA induced no significant T-cell activation, as MPLA signals through TLR-4 in the TRIF-mediated pathway only. However, TLR-4 signalling through MyD88 is essential for the maturation and migration of local DCs loaded with anti-MR conjugates to local

lymph nodes (McKenzie et al., 2007) Therefore, the immunisations were repeated using both MPLA (TLR4-mediated TRIF pathway) and CpG (TLR9-mediated MyD88 pathway) as adjuvants to activate TRP-2 specific, IFN-γ secreting T-cells.

Although this study indicated that targeting using 5D3 induces T-cell activation anti-MR mAbs-TRP-2 conjugates were not considered the ideal tools to test this question as chemical conjugation is an inefficient method of linking Ag. In this way, anti-MR chimeric antibodies were engineered as described in chapter 3 and 4. MR-specific chimeric Abs carry the constant region of mouse IgG in order to avoid xenogenic immune responses. Moreover, the Fc region of the chimeric Abs was mutated to prevent engagement with FcγRs.

In vitro Ag presentation assays were performed using T-cell hybridomas such as MF2, B3Z and 1C5.1, to investigate the capacity of anti-MR chimeric Abs to deliver Ag to APC and induce T-cell proliferation. In our antigen targeting srudies the isotype control always induced T-cell activation even when used for *in vivo* DNA immunisation, which is not consistent with the published data that used the same isotype controls such as IgG1-HEL and IgG1-OVA (Hawiger et al., 2001, Bonifaz et al., 2002).The DNA sequence of our clones encoding isotype control was verified by comparing it with the original sequence obtained from the laboratory of Prof. Michel Nussenweig (Hawiger et al., 2001). Although the Fc portions of the chimeric Abs are mutated they remain able to bind FcγRI and we need to consider the possibility of FcR-mediated uptake/presentation, which is dicussed in detail in section 5.4.2. Murine DCs

and macrophages predominantly express FcγRI, FcγRIIB and FcγRIII (Nimmerjahn and Ravetch, 2008). Regnault et al. reported that Ag uptake by DCs through FcγRs is 100 times more efficient than the soluble Ags. This results in MHC-II as well as MHC-I restricted Ag presentation, and priming of both $CD4^+$ and $CD8^+$ T-cell responses (Regnault et al., 1999).

The chimeric Abs used in this project were tested for their binding to FcγRs expressed on bone marrow-derived macrophages (BM-macrophages). BM-macrophages derived from WT and MR-/- mice were cultured and were collected at day-7 by using 10 mM EDTA in PBS, washed in PBS. MR expression was confirmed by FACS analysis, as shown in figure 7.1. The binding of chimeric Abs to FcγRs expressed on BM-macrophages was tested by the following protocol. 5D3-OVA, IgG1-OVA, 5D3-HEL, IgG1-HEL and anti-DEC205-HEL Abs were labelled with FITC by using Fluoro-Trap fluorescein conjugation kit (InnovaBiosciences) according to the manufacturer's instructions. 50 µl aliquots containing $2x10^5$ macrophages derived from bone marrow of WT and MR-/- mice were distributed in 96 well tissue culture plates. Macrophages were incubated on ice for 30 minutes in FACS buffer in the presence and absence of normal mouse serum (20 % final) and 2.4G2 (Anti-Fc receptor Ab, 20 µg/ml final) respectively. Then 50µl of of labelled antibodies (10µg/ml) were added into the wells containing macrophages and on ice for an hour. Cells were washed 3 times with FACS wash and fixed by using 2% formaldehyde in PBS. Flow cytometry was performed to assess the binding of antibodies to FcγR expressed on macrophages.

Anti-MR chimeric Abs did not show any binding to the macrophages under any condition, as shown in figure 7.2. This is in agreement with earlier studies by Clynes et al. showing that the Fc portion of chimaeric Abs is mutated and therefore they lack the ability to bind FcγRs (Clynes et al., 2000). Surprisingly, 5D3-HEL and 5D3-OVA did not show any binding to MR expressed by the WT macrophages. To determine the cause of this, purified Abs were analysed by SDS-PAGE to test their integrity. It was found that the MR-specific Abs 5D3-HEL and 5D3-OVA (indicated by the red arrows) were degraded almost completely and a major cleaved product of of MW 140 KDa was detected as shown in the figure 7.3. Thus, the degradation of 5D3-HEL or 5D3-OVA can render them unable to bind MR on WT macrophages.

Therefore, it was concluded that T-cells proliferation in response to the isotype controls (IgG1-OVA and IgG1-HEL) was not likely due to FcγR mediated Ag targeting. Hence, the lack of enhanced T-cell proliferation in response to 5D3-OVA and 5D3-HEL could be due to the nature of *in vitro* Ag presentaion assays, which involve the co-culturing of BMDCs and T-cell hybridoma at 37 °C for 18 hours. The anti-MR Abs used in these Ag presentation assays could potentially rapidly degrade due to their labile nature, and the isotype control being stable may persist longer and induce T-cell proliferation. So, the degradation of the 5D3-HEL or 5D3-OVA may be the cause of reduced T-cell proliferation in our Ag targeting studies. The internalisation ability of degraded anti-MR Abs could be further tested by performing an endocytosis assay in the presence of DMA, which inhibits pinocytosis-mediated endocytosis and allows only receptor mediated endocytosis. Moreover, to test the actual potential of

5D3-OVA, a positive control such as DEC205-OVA could be generated to compare in parallel the contribution of both 5D3-OVA and DEC205-OVA with IgG1-OVA to induce T-cell proliferation.

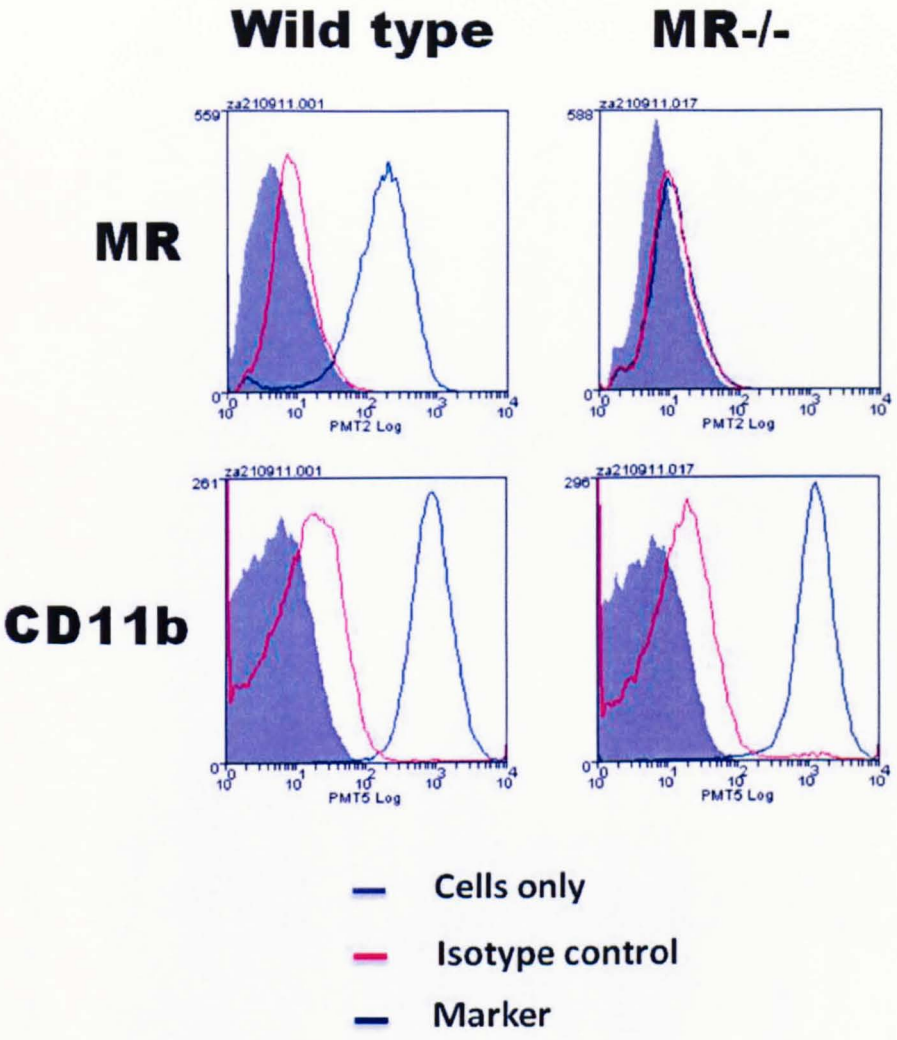


Figure 7.1: Flowcytometric analysis to test MR (CD206) expression on the surface of macrophages derived from the bone marrow of WT and MR-/- mice.

Macrophages derived from mice bone-marrow of WT and MR-/- mice were cultured and on day 7 expression of CD206 (MR), CD11b was analysed by FACS.

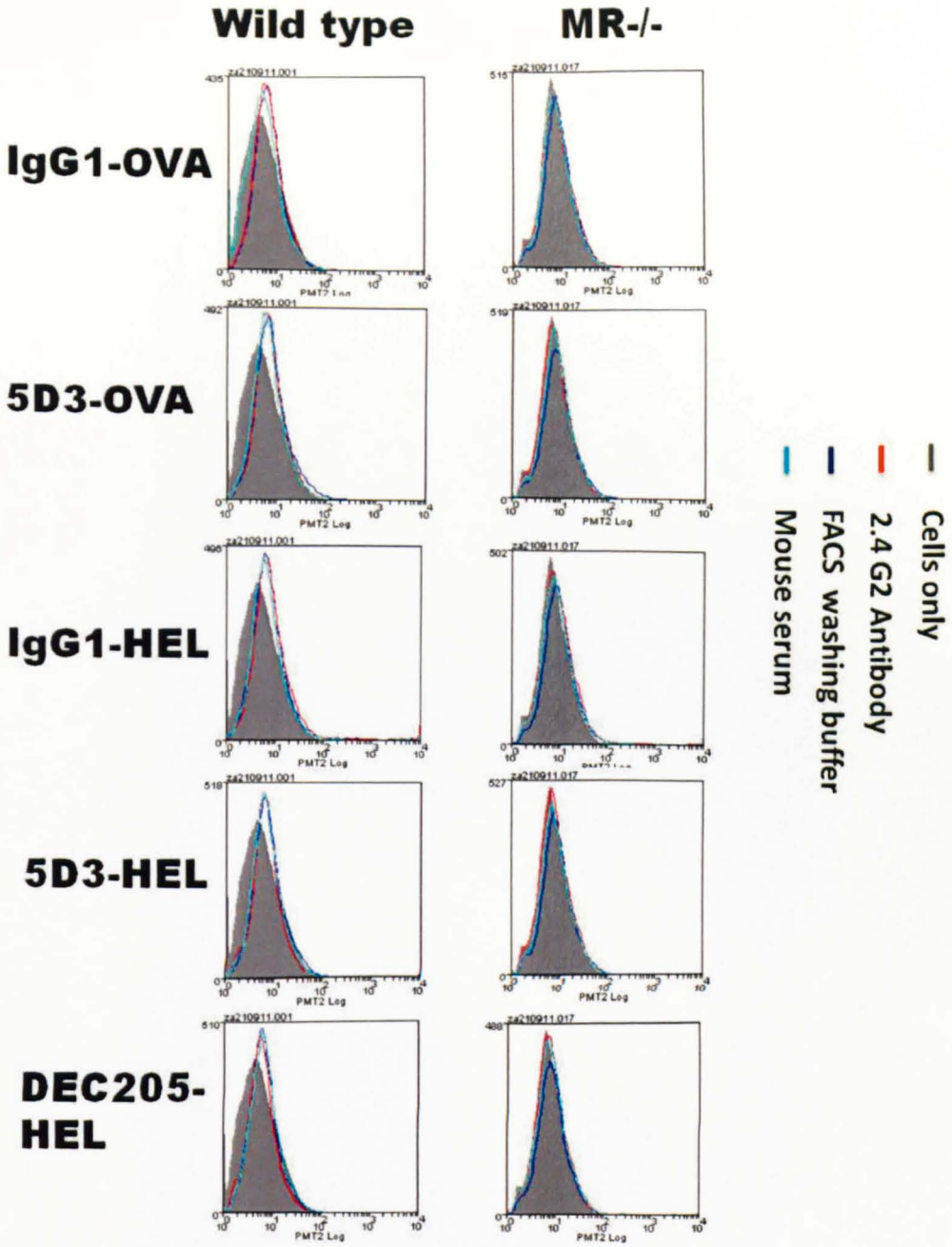


Figure 7.2: Binding assessment of the panel of chimeric antibodies to FcγR expressed on bone-marrow derived macrophages.

Antibodies were labelled with FITC by using Fluoro-Trap fluorescein labelling kit (InnovaBiosciences) by following manufacturer's instructions. $2x10^5$ macrophages derived from WT and MR -/- mice bone-marrow were incubated in the presence and absence of mouse serum or anti-FcRII/III Ab (2.4 G2) followed by the incubation with the labelled antibodies for an hour on ice. After incubation cells were washed with PBS, fixed with 2% formaldehyde and the binding to FcγR was assessed by flow cytometry.

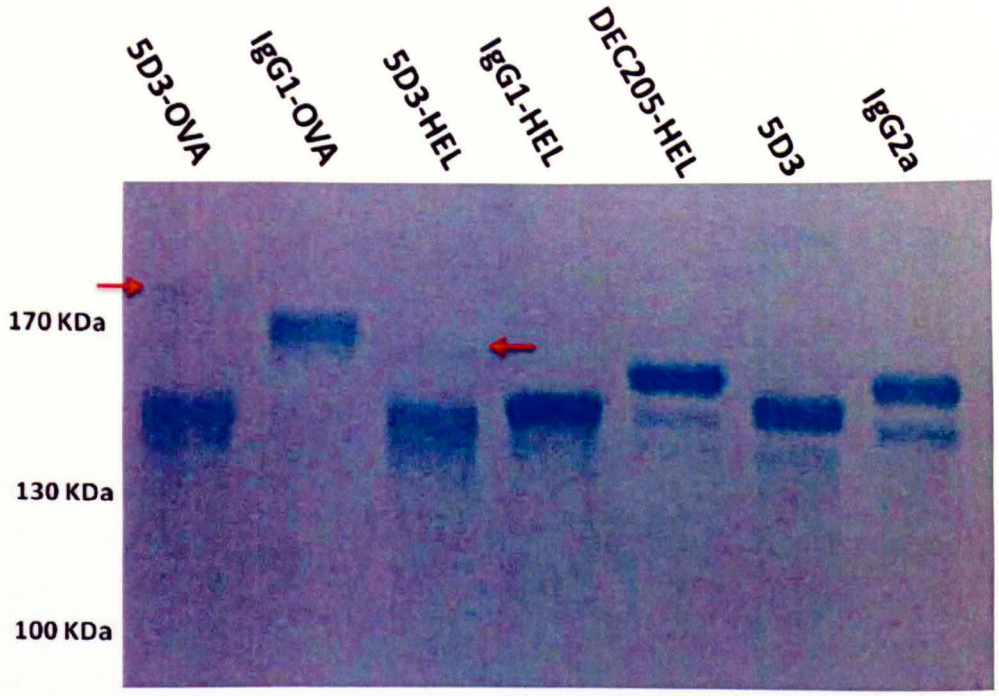


Figure 7.3: SDS-PAGE to check the integrity of panel of antibodies used in the project. 3μg of antibodies were separated in 6% acrylamide gels and stained with Simply Blue safe stain. Red arrows indicate the position of intact 5D3-OVA and 5D3-HEL Abs.

In the future, anti-MR chimeric Abs would be used to prepare lower molecular weight antibody fragments in order to avoid Abs degradation and to enhance their distribution when immunised *in vivo*, in collaboration with Dr. Jordi Ochando (Mount Sinai School of Medicine). Small engineered fragments of the Ab include single chain-Fv fragment (Sc-Fv), dimers of Sc-Fv (diabody) and minibodies which is a pair of Sc-FV fused to single constant domain CH3 $(ScFv\text{-}CH3)^2$ (Wu and Senter, 2005).The expression of low molecular weight antibody fragments Sc-Fv (25KDa) and dibodies (55KDa) is relatively low, and they are less soluble and highly prone to aggaregation compared to full length Abs and minibodies (Holliger and Hudson, 2005). Moreover, minibodies are more stable than Sc-Fv and some minibodies such as Her2 (Pettersen et al., 2004) and CEA are in their preclinical stages for tumour

imaging and therapies. Therefore, minibodies fused with the model Ag could be a better option for purposes of Ag targeting.

7.3 Use of glycopolymers for Ag targeting to APC

Different approaches used for antigen targeting through MR to induce immunity involve Ab-mediated targeting (He et al., 2007) or natural ligands. Ligand mediated Ag targeting has been successful, for example oxidized mannan linked with the MUC1 antigen is in phase III of clinical trials to treat patients with stage II breast cancer as discussed in section 6.4.

We used glycopolymers containing different relative percentages of mannose and galactose. Glycopolymers bind MR and are a better choice for antigen delivery and vaccine development compared to other methods (as mentioned above) because they are easily manipulated to change the content of mannose monomers, which directly affects the binding properties of the glycopolymers to lectin receptors. Therefore, *libraries* of glycopolymers having different bidning kinetics can be generated and tested. It has been established that glycopolymers or carbohydrates, having high off rate or dissociation rate are more potent for Ag delivery and cross-presentation, which is a critical mechanism involved in the development of vaccines against cancer and other infections requiring CTL-based immunity (Howland and Wittrup, 2008). Synthesis of the glycopolymers employs simple click chemistry and is relatively easy and less expensive in a large scale. Singh et al. reported that OVA linked with the glycans 3-sulfo-Lewis^A or N-acetylglucosamine targets DCs specifically through MR and enhances cross-presentation of OVA (Singh

et al., 2011). Therefore, to avoid binding of glycopolymers to multiple receptors the glycopolymers containing 3-sulfo-Lewis^A or N-acetylglucosamine monomers could be prepared using the same methodology (click chemistry) to facilitate antigen delivery specifically through MR.

7.4 Relevance of our antigen targeting studies

Our MR-mediated Ag targeting studies help to understand the importance of adjuvants, type or mode of vaccination, and the use of glycopolymers in MR-targeted vaccines, as described below in detail.

7.4.1 Role of adjuvants

Results obtained by the immunisation of 5D3-TP2 antibodies indicate the importance of MyD88 signaling in the activation of TRP-2 specific T-cells. In this study, MPLA, which signals through TLR-4 via TRIF pathway alone did not induce T-cell activation. CpG (signalling through TLR-9 via Myd88 pathway) in combination with MPLA, however, effectively induced TRP-2 specific T-cell activation. Mckenzie et al. reported that TLR-4 mediated MyD88 signaling is essential for the maturation and migration of local DCs loaded with Anti-MR Ab to local lyph nodes (McKenzie et al., 2007), demonstrating its importance in the induction of Ag specific immunity. Moreover, MyD88 signaling recruits TAP to early endosomes, which is believed to be involved in the transfer of peptide and allows loading on the MHC-I molecule in the early endosome, leading to cross-presentation of OVA (Burgdorf et al., 2008). However, Singh et al. reported that OVA linked to glycans such as 3-sulfo-Lewis^A and N-acetylglucosamine can target OVA to

MR and result in the cross-presentation of OVA without TLR-4 signaling through the MyD88 pathway (Singh et al., 2011). Obviously, the generation of highly specific MR-targeting tools will help in defining the potential of MR as a target for Ag delivery.

7.4.2 Method of vaccination (Protein versus DNA)

In our studies DNA vaccination was highly effective in activating Ag specific $CD8^+$ T-cells. Enhanced T-cell responses achieved by DNA immunisation could be due to its ability to exploit two pathways of antigen presentation. Firstly, due to the direct Ag presentation through MHC-I when DNA is transfected into local DCs. Secondly, DNA taken up by the somatic cells could be translated and chimeric Abs secreted out of the cells at the site of immunisation, which could be taken up by local DCs through either MR or FcγRI, resulting in cross-presentation of Ag (Pudney et al., 2010). When immunized, chimeric Abs (protein) induce relatively low T-cell activation as they only target APCs, and excludes the possibility to exploit non APCs for Ag production. However, DNA immunisation exploits both APC and non APC for Ag expression and presentation. Cho et al. reported that DNA immunised into non APCs induced more rapid and vigorous $CD8^+$ T-cells proliferation as compared to APC: first, due to relatively more numbers of non APCs and, second, due to the relatively prolonged expression of DNA in non APC as compared to APC (Cho et al., 2001).

7.4.3 Effectivenes of the *in vitro* versus *in vivo* assays to test MR targeting tools

According to our studies *in vitro* Ag presentations assay might not be an effective method to test MR-targeted vaccines due to the presence of sMR in the culture medium. sMR can bind directly to anti-MR antibodies or MR ligands. In this way, it could block or reduce the binding of Ag targeting reagents to DCs, thereby effecting Ag presentation (Jordens et al., 1999). Moreover, collagen fragments could be present in the serum that can also block MR and inhibit Ag presentation. Due to these limitations, *in vivo* immunisation could be be more appropriate to test anti-MR Abs. He et al. reported that *in vivo* immunisation of human anti-MR Ab (B11) fused with OVA (B11-OVA) along with the appropriate adjuvants can induce OVA specific $CD4^+$ and $CD8^+$ T-cell responses (He et al., 2007). Additionally, because of the restricted expression of MR in DCs *in vivo* could better predict the efficacy of MR-targeting.

7.4.4 Comaprision of the mAbs and glycpolymers for Ag delivery

In our experience glycopolymers seem a better choice compared to mAbs for the targeting of Ag to APCs and the induction of immunity, for the following reasons. First, generation of antibodies against different receptors is an expensive, difficult and time consuming process. Moreover, chimeric antibodies have low expression and sometimes they become labile due to genetic modification. Second, antibodies used for Ag targeting can induce adverse immune responses when immunised in humans due to xenogenic effect, while these effects could be avoided using natural ligands or

glycopolymers for targeting. Due to these limitations, glycopolymers provide a more robust and convenient alternative method for antigen delivery to APCs. Synthesis of the glycopolymers employs simple click chemistry, making it relatively easy and less inexpensive to synthesise glycopolymers on a large scale. Moreover, glycopolymers can be easily manipulated to change the content of mannose monomers, which directly affects the binding properties of glycpolymers to lectin receptors. Therefore, libraries of glycopolymers having different binding kinetics can be generated using different quantities of mannose monomers; however. This methodology is highly novel and will be further investigated in the lab.

7.5 Potential Applications of MR targeting tools

Previous studies showed that antigens derived from common infectious diseases such as the circumsporozite protein of malaria parasite (Boscardin et al., 2006), gig protein of HIV (Bozzacco et al., 2007) and nuclear antigen of Epstein-Barr virus (Gurer et al., 2008) can be targeted through DEC-205 in the presence of an adjuvant and result in strong antigen specific immunity. Recently Tsuji et al., found that DCs targeting through MR in the presence of an appropriate adjuvant leads to cross-presentation and induction of powerful CTL immunity to facilitate immunotherapy of cancer. In his study, MR specific antibodies fused to cancer antigen NY-ESO-1 were used to target DC specifically through MR. This resulted in the induction of antigen specific $CD8^+$ and $CD4^+$ T-cells, which makes it a promising approach to develop strong immunity against cancer (Tsuji et al., 2010). Anti-MR reagents

generated in this study could be used to target malarial, cancerous and viral Ags once their potential has been established using *in vitro* and *in vivo* assays. On the other hand, in the absence of an infectious signal, antigen targeting through DEC-205 may lead to antigen specific T-cell tolerance. In this respect, ß cell antigen targeting results in the deletion of autoreactive CD8+ T-cells, which causes Type 1 Diabetes Mellitus (Mukhopadhaya et al., 2008). In previous studies the role of MR in homeostasis and allergy has been established. Recently Emara et al., demonstrated the binding of major allergens such as Der p-1 and Fel d 1 to MR and showed that MR binding promotes Th2 responses indicating a role in allergy and in modulation of T-cell differentiation (Emara et al., 2011, Royer et al., 2010). Therefore, in future, the anti-MR reagents generated in this study could be used to target allergens and self-Ags to APC in an attempt to induce tolerance.

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9 Appendices

Appendix 1: DEC205-HEL Heavy chain

EcoRI.5′tgggatggtcatgtatcatccttttctagtagcaactgcaactggag
tacattcaGAGGTGAAGCTGTTGGAATCTGGAGGAGGTTTGGTACAGCCGGGGGGT
TCTCTGAGACTCTCCTGTGCAGCTTCTGGATTCACCTTCAATGATTTCTACATGAA
CTGGATCCGCCAGCCTCCAGGGCAGGCACCTGAGTGGTTGGGTGTTATTAGAAACA
AAGGTAATGGTTACACAACAGAGGTCAATACATCTGTGAAGGGGCGGTTCACCATC
TCCAGAGATAATACCCAAAACATCCTCTATCTTCAAATGAACAGCCTGAGAGCTGA
GGACACCGCCATTTACTACTGTGCAAGAGGCGGTCCTTATTACTACAGTGGTGACG
ACGCCCCTTACTGGGGCCAAGGAGTCATGGTCACAGTCTCCTCAgccaccaccaag
ggcccatctgtctatccactggcccctggatctgctgcccaaactaactccatggt
gaccctgggatgcctggtcaagggctatttccctgagccagtgacagtgacctgga
actctggatccctgtccagcggtgtgcacaccttcccagctgtcctgcagtctgac
ctctacactctgagcagctcagtgactgtcccctccagcacctggcccagcgagac
cgtcacctgcaacgttgcccacccggccagcagcaccaaggtggacaagaaaattg
tgcccagggattgtggttgtaagccttgcatatgtacagtcccagaagtatcatct
gtcttcatcttccccccaaagcccaaggatgtgctcaccattactctgactcctaa
ggtcacgtgtgttgtggtagcaatcagcaaggatgatcccgaggtccagttcagct
ggtttgtagatgatgtggaggtgcacacagctcagacgcaaccccgggaggagcag
ttcaacagcactttccgctcagtcagtgaacttcccatcatgcaccaggactggct
caatggcaaggagttcaaatgcagggtcaacagtgcagctttccctgcccccatcg
agaaaaccatctccaaaaccaaaggcagaccgaaggctccacaggtgtacaccatt
ccacctcccaaggagcagatggccaaggataaagtcagtctgacctgcatgataac
agacttcttccctgaagacattactgtggagtggcagtggaatgggcagccagcgg
agaactacaagaacactcagcccatcatggacacagatggctcttacttcgtctac
agcaagctcaatgtgcagaagagcaactgggaggcaggaaatactttcacctgctc
tgtgttacatgagggcctgcacaaccaccatactgagaagagcctctcccactctc
ctggtaaagctagcGACATGGCCAAGAAGGAGACAGTCTGGAGGCTCGAGGAGTTC
GGTAGGTTCACAAACAGGAACACAGACGGTAGCACAGACTATGGTATTCTCCAGAT
TAACAGCAGGTATTATGACGGTAGGACATGATAG 3′-NotI

Appendix 2: DNA sequence of DEC205-HEL Light chain

EcoRI-

5′atgggatggtcatgtatcatccttttctagtagcaactgcaactggagtacat
tcaGACATCCAGATGACACAGTCTCCGTCATTTCTGTCTACATCTCTTGGAAACAG
CATCACCATCACTTGCCATGCCAGTCAGAACATCAAGGGTTGGTTAGCCTGGTACC
AACAAAAGTCAGGGAATGCTCCTCAACTGTTGATTTATAAGGCATCTAGCCTGCAA
TCAGGGGTTCCATCAAGATTCAGTGGCAGTGGATCTGGAACAGATTATATTTTCAC
TATCAGCAACCTACAGCCTGAAGATATTGCCACTTATTACTGTCAGCATTATCAAA
GCTTTCCGTGGACGTTCGGTGGAGGCACCAAGCTGGAATTGAAACGGgctgatgct
gcaccaactgtatccatcttcccaccatccagtgagcagttaacatctggaggtgc
ctcagtcgtgtgcttcttgaacaacttctaccccaaagacatcaatgtcaagtgga
agattgatggcagtgaacgacaaaatggcgtcctgaacagttggactgatcaggac
agcaaagacagcacctacagcatgagcagcaccctcacgttgaccaaggacgagta
tgaacgacataacagctatacctgtgaggccactcacaagacatcaacttcaccca
ttgtcaagagcttcaacaggaatgagtgttga-3′ NotI.

Appendix-3 DNA sequence of 5D3-HEL heavy chain.

ATGGACAGGCTTACTTCCTCATTCCTACTGCTGATTGTCCCTGCATATGTCCTGTC
TCAGGTTACTCTGAAAGAGTCTGGCCCTGGGATATTGCAGCCCTCCCAGACCCTCA
GTCTGACTTGCACTTTCTCTGGGTTTTCACTGAGCACTTATGGTATGGGTGTGGGC
TGGATTCGTCAGCCTTCAGGGAAGGGTCTGGAGTGGCTGGCAAACATTTGGTGGGA
TGATGGTAAGTACTACAATCCATCTCTGAAAAACCGGCTCACAATCTCCAAGGACA
CCTCCAACAATCAAGCATTCCTCAAGATCACCAATGTGGACACTGTAGATACTGCC
GCATACTACTGTTTTCGGATTCCCTTTTATTATCATGATGCGGATCACTGGGGCCA
AGGCACTCTGGTCACTGTCTCTTCAGCTGAAACAACGGGCCCATCTGTCTATCCAC
TGGCCCCTGGATCTGCTGCCCAAACTAACTCCATGGTGACCCTGGGATGCCTGGTC
AAGGGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACTCTGGATCCCTGTCCAG
CGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACTCTGAGCAGCT
CAGTGACTGTCCCCTCCAGCACCTGGCCCAGCGAGACCGTCACCTGCAACGTTGCC
CACCCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGTGGTTG
TAAGCCTTGCATATGTACAGTCCCAGAAGTATCATCTGTCTTCATCTTCCCCCCAA
AGCCCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTA
GCAATCAGCAAGGATGATCCCGAGGTCCAGTTCAGCTGGTTTGTAGATGATGTGGA
GGTGCACACAGCTCAGACGCAACCCCGGGAGGAGCAGTTCAACAGCACTTTCCGCT
CAGTCAGTGAACTTCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTTCAAA
TGCAGGGTCAACAGTGCAGCTTTCCCTGCCCCCATCGAGAAAACCATCTCCAAAAC
CAAAGGCAGACCGAAGGCTCCACAGGTGTACACCATTCCACCTCCCAAGGAGCAGA
TGGCCAAGGATAAAGTCAGTCTGACCTGCATGATAACAGACTTCTTCCCTGAAGAC
ATTACTGTGGAGTGGCAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAACACTCA
GCCCATCATGGACACAGATGGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAGA
AGAGCAACTGGGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACATGAGGGCCTG
CACAACCACCATACTGAGAAGAGCCTCTCCCACTCTCCTGGTAAAGCTAGCGACAT
GGCCAAGAAGGAGACAGTCTGGAGGCTCGAGGAGTTCGGTAGGTTCACAAACAGGA
ACACAGACGGTAGCACAGACTATGGTATTCTCCAGATTAACAGCAGGTATTATGAC
GGTAGGACATGATAG

Appendix-4 DNA sequence of 5D3-HEL light chain.

ATGGCCATGAAGACGCCTGCCCAGGCATTGGCAATTTGGTTACTCTGGGTCTCAGG
TGCCAGATGTGATATTCAAGTGACTCAATCTCCATCCTCCCTCTTGGCATCTCTAG
GAGAGAGAGTCACTATCACATGCCAGACAAGTCAGACCATTAGCAAAAACCTAAAC
TGGTATCAGCAGAAACCAGGACAAGTTCCTATACTCTTGATCTATTTTGCAACCAG
TTTGCAGACTGGCATGCCATCAAGGTTCAGTGGCCAATATTCTGGGAGAAGTTTCA
CTCTAACCATCACTAGCCTGGAACCAGAAGATATTGCAAATTATTTTTGTCTGCAG
CATTACAATCCTCCGTGGACGTTCGGTGGAGGGACCAAGCTGGAATTGAAACGGGC
TGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATATG
GAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACATCAATGTC
AAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTGA
TCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACCAAGG
ACGAGTATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACT
TCACCCATTGTCAAGAGCTTCAACAGGAATGAGTGTTGA

Appendix-5 DNA sequence of 5D3-OVA heavy chain.

ATGGACAGGCTTACTTCCTCATTCCTACTGCTGATTGTCCCTGCATATGTCCTGTC
TCAGGTTACTCTGAAAGAGTCTGGCCCTGGGATATTGCAGCCCTCCCAGACCCTCA
GTCTGACTTGCACTTTCTCTGGGTTTTCACTGAGCACTTATGGTATGGGTGTGGGC
TGGATTCGTCAGCCTTCAGGGAAGGGTCTGGAGTGGCTGGCAAACATTTGGTGGGA
TGATGGTAAGTACTACAATCCATCTCTGAAAAACCGGCTCACAATCTCCAAGGACA
CCTCCAACAATCAAGCATTCCTCAAGATCACCAATGTGGACACTGTAGATACTGCC
GCATACTACTGTTTTCGGATTCCCTTTTATTATCATGATGCGGATCACTGGGGCCA
AGGCACTCTGGTCACTGTCTCTTCAGCTGAAACAACGGGCCCATCTGTCTATCCAC
TGGCCCCTGGATCTGCTGCCCAAACTAACTCCATGGTGACCCTGGGATGCCTGGTC
AAGGGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACTCTGGATCCCTGTCCAG
CGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACTCTGAGCAGCT
CAGTGACTGTCCCCTCCAGCACCTGGCCCAGCGAGACCGTCACCTGCAACGTTGCC
CACCCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGTGGTTG
TAAGCCTTGCATATGTACAGTCCCAGAAGTATCATCTGTCTTCATCTTCCCCCCAA
AGCCCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTA
GCAATCAGCAAGGATGATCCCGAGGTCCAGTTCAGCTGGTTTGTAGATGATGTGGA
GGTGCACACAGCTCAGACGCAACCCCGGGAGGAGCAGTTCAACAGCACTTTCCGCT
CAGTCAGTGAACTTCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTTCAAA
TGCAGGGTCAACAGTGCAGCTTTCCCTGCCCCCATCGAGAAAACCATCTCCAAAAC
CAAAGGCAGACCGAAGGCTCCACAGGTGTACACCATTCCACCTCCCAAGGAGCAGA
TGGCCAAGGATAAAGTCAGTCTGACCTGCATGATAACAGACTTCTTCCCTGAAGAC
ATTACTGTGGAGTGGCAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAACACTCA
GCCCATCATGGACACAGATGGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAGA
AGAGCAACTGGGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACATGAGGGCCTG
CACAACCACCATACTGAGAAGAGCCTCTCCCACTCTCCTGGTAAAGCTAGCATGTT
GGTGCTGTTGCCTGATGAAGTCTCAGGCCTTGAGCAGCTTGAGAGTATAATCAACT
TTGAAAAACTGACTGAATGGACCAGTTCTAATGTTATGGAAGAGAGGAAGATCAAA
GTGTACTTACCTCGCATGAAGATGGAGGAAAAATACAACCTCACATCTGTCTTAAT
GGCTATGGGCATTACTGACGTGTTTAGCTCTTCAGCCAATCTGTCTGGCATCTCCT
CAGCAGAGAGCCTGAAGATATCTCAAGCTGTCCATGCAGCACATGCAGAAATCAAT
GAAGCAGGCAGAGAGGTGGTAGGGTCAGCAGAGGCTGGAGTGGATGCTGCA

Appendix-6 DNA sequence of IgG1-HEL Heavy chain

ATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAACTGCAACTGGAGTACATTC
ACAGGTGCAGCTGAAAGAGTCAGGACCTGGTCTGGTGCAGCCCTCACAGACCCTGT
CTCTCACCTGCACTGTCTCTGGGTTCTCACTAATCAGCTATCATGTAACCTGGGTT
CGCCAGCCTCCTGGAAAGAGTCTGGTGTGGATGGGAACAATATGGACTGGTGGAGG
TAGAAATTATAATTCGGCTGAACAATCCCGACTGAGCATCAGCCGGGACACCTCCA
AGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCAACCTGAAGACACAGGCACTTAC
TACTGTGCCAGACATCGAGGGGGGTATAACTACGGCTTTGATTACTGGGGCCAAGG
AGTCATGGTCACAGTCTCCTCAGCCACCACCAAGGGCCCATCTGTCTATCCACTGG
CCCCTGGATCTGCTGCCCAAACTAACTCCATGGTGACCCTGGGATGCCTGGTCAAG
GGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACTCTGGATCCCTGTCCAGCGG
TGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACTCTGAGCAGCTCAG
TGACTGTCCCCTCCAGCACCTGGCCCAGCGAGACCGTCACCTGCAACGTTGCCCAC
CCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGTGGTTGTAA
GCCTTGCATATGTACAGTCCCAGAAGTATCATCTGTCTTCATCTTCCCCCCAAAGC
CCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTAGCA
ATCAGCAAGGATGATCCCGAGGTCCAGTTCAGCTGGTTTGTAGATGATGTGGAGGT
GCACACAGCTCAGACGCAACCCCGGGAGGAGCAGTTCAACAGCACTTTCCGCTCAG
TCAGTGAACTTCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTTCAAATGC
AGGGTCAACAGTGCAGCTTTCCCTGCCCCCATCGAGAAAACCATCTCCAAAACCAA
AGGCAGACCGAAGGCTCCACAGGTGTACACCATTCCACCTCCCAAGGAGCAGATGG
CCAAGGATAAAGTCAGTCTGACCTGCATGATAACAGACTTCTTCCCTGAAGACATT
ACTGTGGAGTGGCAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAACACTCAGCC
CATCATGGACACAGATGGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAGAAGA
GCAACTGGGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACATGAGGGCCTGCAC
AACCACCATACTGAGAAGAGCCTCTCCCACTCTCCTGGTAAAGCTAGCGACATGGC
CAAGAAGGAGACAGTCTGGAGGCTCGAGGAGTTCGGTAGGTTCACAAACAGGAACA
CAGACGGTAGCACAGACTATGGTATTCTCCAGATTAACAGCAGGTATTATGACGGT
AGGACATGATAG

Appendix-7 DNA sequence of IgG1-OVA Heavy chain

ATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAACTGCAACTGGAGTACATTC
ACAGGTGCAGCTGAAAGAGTCAGGACCTGGTCTGGTGCAGCCCTCACAGACCCTGT
CTCTCACCTGCACTGTCTCTGGGTTCTCACTAATCAGCTATCATGTAACCTGGGTT
CGCCAGCCTCCTGGAAAGAGTCTGGTGTGGATGGGAACAATATGGACTGGTGGAGG
TAGAAATTATAATTCGGCTGAACAATCCCGACTGAGCATCAGCCGGGACACCTCCA
AGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCAACCTGAAGACACAGGCACTTAC
TACTGTGCCAGACATCGAGGGGGGTATAACTACGGCTTTGATTACTGGGGCCAAGG
AGTCATGGTCACAGTCTCCTCAGCCACCACCAAGGGCCCATCTGTCTATCCACTGG
CCCCTGGATCTGCTGCCCAAACTAACTCCATGGTGACCCTGGGATGCCTGGTCAAG
GGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACTCTGGATCCCTGTCCAGCGG
TGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACTCTGAGCAGCTCAG
TGACTGTCCCCTCCAGCACCTGGCCCAGCGAGACCGTCACCTGCAACGTTGCCCAC
CCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGTGGTTGTAA
GCCTTGCATATGTACAGTCCCAGAAGTATCATCTGTCTTCATCTTCCCCCCAAAGC
CCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCACGTGTGTTGTGGTAGCA
ATCAGCAAGGATGATCCCGAGGTCCAGTTCAGCTGGTTTGTAGATGATGTGGAGGT
GCACACAGCTCAGACGCAACCCCGGGAGGAGCAGTTCAACAGCACTTTCCGCTCAG
TCAGTGAACTTCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTTCAAATGC
AGGGTCAACAGTGCAGCTTTCCCTGCCCCCATCGAGAAAACCATCTCCAAAACCAA
AGGCAGACCGAAGGCTCCACAGGTGTACACCATTCCACCTCCCAAGGAGCAGATGG
CCAAGGATAAAGTCAGTCTGACCTGCATGATAACAGACTTCTTCCCTGAAGACATT
ACTGTGGAGTGGCAGTGGAATGGGCAGCCAGCGGAGAACTACAAGAACACTCAGCC
CATCATGGACACAGATGGCTCTTACTTCGTCTACAGCAAGCTCAATGTGCAGAAGA
GCAACTGGGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACATGAGGGCCTGCAC
AACCACCATACTGAGAAGAGCCTCTCCCACTCTCCTGGTAAAGCTAGCATGTTGGT
GCTGTTGCCTGATGAAGTCTCAGGCCTTGAGCAGCTTGAGAGTATAATCAACTTTG
AAAAACTGACTGAATGGACCAGTTCTAATGTTATGGAAGAGAGGAAGATCAAAGTG
TACTTACCTCGCATGAAGATGGAGGAAAAATACAACCTCACATCTGTCTTAATGGC
TATGGGCATTACTGACGTGTTTAGCTCTTCAGCCAATCTGTCTGGCATCTCCTCAG
CAGAGAGCCTGAAGATATCTCAAGCTGTCCATGCAGCACATGCAGAAATCAATGAA
GCAGGCAGAGAGGTGGTAGGGTCAGCAGAGGCTGGAGTGGATGCTGCATAA

Appendix-8 DNA sequence of IgG1-HEL Light chain

ATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAACTGCAACTGGAGTACATTC
AGACATCCAGATGACCCAGTCTCCTTCACTCCTGTCTGCATCTGTGGGAGACAGAG
TCACTCTCAACTGCAAAGCAAGTCAGAATATTAATAAGAACTTAGACTGGTATCAG
CAAAAGCTTGGAGAAGCGCCAAAAGTCCTGATATATTATACAGACAATTTGCAAAC
GGGCTTCTCATCAAGGTTCAGTGGCAGTGGATCTGGTACAGATTACACACTCACCA
TCAGCAGCCTGCAGCCTGAAGATGTTGCCACATATTACTGCTATCAGTATAACAGT
GGGCCCACGTTTGGACCTGGGACCAAGCTGGAACTGAAACGGGCTGATGCTGCACC
AACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTGCCTCAG
TCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACATCAATGTCAAGTGGAAGATT
GATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAA
AGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACCAAGGACGAGTATGAAC
GACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCCATTGTC
AAGAGCTTCAACAGGAATGAGTGTTGA