



Synthetic glyco-viruses for targeted DNA vaccination vectors

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Abstract

Viruses are powerful vectors for the delivery of nucleic acids, with applications in gene therapy and vaccination. However, major challenges for this technology include mis-targeting of the vector and neutralization by host antibody responses. Here we show that chemical addition of synthetic glycans to adenovirus (Ad) increased resistance to neutralizing antibodies and other critical host clearance mechanisms. Carbohydrate ligands were synthesised and functionalised with a cyanol methyl thiol group, which was subsequently activated to the amine reactive 2-imino-2-methoxyethyl (IME). Ads were coated with ligand whilst maintaining virus cogency, with up to 58% of total Ad lysine residues glycosylated. The viral tropism of glyco-Ads was switched to target macrophages and dendritic cells (DCs), with high selectivity for the complementary sugar receptors. In vaccination studies, DC targeted glyco-virus enhanced antigen-specific T cell responses. Thus, chemical glycosylation of the Ad capsid allows modulation of tropism and shielding from sequestration and neutralizing antibodies. Since manipulation of this process is facile, it provides a flexible and potentially universal solution to key obstacles facing the utilization of viral vectors in therapeutic and vaccination contexts.

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Abbreviations

APC	Antigen presenting cell
Ac	Acetyl
Ad	Adenovirus
APC	Allophycocyanin
Arg	Arginine
Asp	Aspartic acid
BMDC	Bone marrow derived dendritic cells
BSA	Bovine serum albumin
CAN	Ceric ammonium nitrate
CAR	Coxsackievirus and adenovirus receptor
CBA	Cytokine bead array
CD	Cluster differentiation
CFSE	Carboxy fluorescein succinimidyl ester
COSY	Homonuclear correlation spectroscopy
CpG	– C – phosphate – G –
CRD	Carbohydrate recognition domain
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
DC-SIGN	Dendritic cell
DCM	Dichloromethane
DMAP	4-Dimethylaminopyridine
DMEM	Modification of Basal Medium Eagle
DMF	N, N-dimethylformamide
ELISA	The enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fc	Fragment, crystallisable
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Fuc	Fucose
FX	Blood factor X
Gal	Galactose
GFP	Green fluorescent protein
Gly	Glycine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEL	Hen egg lysozyme
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HSPG	Heparin sulfate proteoglycans
HSQC	heteronuclear single quantum correlation
IL	Interleukin
IM	Intramuscular
IMDM	Iscove's Modified Dulbecco's Medium
IME	2-imino-2-methoxyethyl-1-thioglycoside
INFγ	Interferon gamma
LPS	Lipopolysaccharide
Lys	Lysine
M-CSF	macrophage colony- stimulating factor
Man	Mannose
ManLAM	Mannosylated Lipoarabinomannan
MCP-1	Monocyte chemoattractant protein-1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MR	Mannose receptor
NHS	Hydroxysuccinimide
NLS	Nuclear localization sequence
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethylene glycol
PGE2	Prostaglandin E2
PMB	Para-methoxybenzyl
RANTES	Regulated on activation normal T cell expressed and secreted
RPMI	Roswell Park Memorial Institute medium- 1640
TAPS	N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid
TBDMSCl	tert-Butyldimethylsilyl chloride
TCA	Trichloroacetonitrile
TCR	T cell receptor
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TGF	TGF
Th	Helper T cell
Thr	Threonine
TLR	Toll-like receptors
TNF	Tumor necrosis factors

1. Introduction

1.1 A brief history of vaccination

The first documented case of an attempt to protect a population using inoculation dates from 17th century China. Zhang Lu, in his medical book, described variolation - a technique involving exposure of the patient to material taken from a recently infected person; methods included inserting cotton imbued with pox pus or blowing powdered pox scab into the nose of the child to be inoculated¹. A century later, Francis Home demonstrated the successful inoculation of children against measles, through exposure to infectious skin samples and blood². Edward Jenner, a contemporary of Home, started to record his observations that cowpox, a relatively mild disease, conferred immunity to smallpox. Jenner published his findings in his *Inquiry into the causes and affects of the variolae vaccinae* 1798, which included the famous experiment conducted on a son of a servant, whereby Jenner purposely infected him with cowpox. The term employed by Jenner, 'vaccine inoculation' was used to describe the procedure, later known as 'vaccination'. In the 1800's Louis Pasteur observed that an initial infection of many diseases, including anthrax, left the surviving animal or person in a state of insusceptibility to further infection with the same pathogen. He later applied this principle by developing a vaccine against chicken cholera, in collaboration with Henry Toussaint. In 1879 they developed a strain of the pathogen with attenuated virulence, which offered the chickens some protection against the true cholera bacteria³. This is cited as the first laboratory vaccine. Pasteur proposed his 'exhaustion theory' in which immunity was a consequence of the non-virulent stain of the microbe sequestering all of the nutrients required by the virulent microbe. According to the exhaustion theory, living, non-virulent stains of pathogens are required to maintain immunization. Pasteur went on to demonstrate his

theory with attenuated strains of anthrax, in a series of experiments known as the 'Pouilly-le-fort-experiments'. In 1880, Toussaint went on to develop his own technique of vaccine production, also based on Pasteur's concept of attenuated virulence. Toussaint took blood from sheep that had recently succumbed to anthrax infection and heated it at 55°C for 10 minutes. He then used these heat-treated samples to inoculate other sheep, providing protection against subsequent infections and resulting in fewer anthrax related sheep deaths. Toussaint hypothesised that vaccination made the lymph nodes impermeable to bacteria in some manner⁴. We now understand that both this theory and Pasteur's 'exhaustion theory' are incorrect, however these pioneering works mark the start of the science of vaccination. The use of whole-organism vaccines, either live or synthetically attenuated, represents the first generation of vaccines.

Concurrent to work on whole pathogens, researchers were also attempting to conduct inoculations with purified microbial toxins. Friedrich Loeffler successfully cultured diphtheria bacillus in 1884 and, through the observation that the bacillus remained remote from the histopathological site, he deduced that the bacilli were secreting a toxin⁵. This was later confirmed by the work of Roux and Yersin and in 1890 von Behring and Kitasato demonstrated that specific antitoxin serum could be generated in animals given a sub-lethal dose of the toxin^{6,7}. During the subsequent years researchers started to investigate the composition of serum. Han Buchner is credited with the development of the humoral theory of immunology, so named as it involved substances found in the humours (body fluids). In his 1889 work he first described protective substances in the blood serum capable of killing microorganisms⁸. In 1895 Jules Bordet isolated a heat stable fraction of the sera, after heating at 56°C, which he found agglutinated live bacteria but did not lyse them⁹. Bordet later described the first antibacterial, heat-labile serum component in 1896¹⁰. Antitoxin sera generated in horses and sheep was then used to inoculate children in Paris, with some success. In 1897, Paul Ehrlich opened an immunity unit within a hospital for the standardisation of diphtheria

antitoxin; marking the first standardisation of any biological product¹¹. Ehrlich, inspired by his work on sera, also went on to first propose the antibody formation theory, known as the 'side-chain theory', c.1900 (**Figure 1**). Ehrlich proposed that a series of chemical reactions could describe an organism's reaction to bacterial toxins. He described chemical side chains, produced by cells, which bind to a toxin, negatively affecting its function. These 'side chains' are regenerated once the host has survived the toxin, resulting in the effective immunisation of the host. Ehrlich expanded his theory to include the role of an additional complementary molecule that interacts between the toxin and the side chain. Today we understand that the 'side chains' proposed by Ehrlich are antibodies. John Marrack described the first biochemical properties of the antigen-antibody binding interaction in his book published in 1938^{12,13}. Protein-based vaccines represent the beginnings of the second generation of vaccines. In place of a live organism, using a subunit or a protein associated with a pathogen reduced some of the risks associated with live vaccination with a pathogen.



Figure 1 – Prof. Paul Ehrlich in his Frankfurt Office c. 1900

Ramon reported the first use of an adjuvant during a vaccination in 1925. He noted that the highest titres of circulating antibodies were obtained from animals which had developed an abscess of inflammation at

the site of inoculation^{14,15}. One year on, Alexander Thomas Glenny increased the effectiveness of diphtheria toxoid vaccination by first treating the toxoid by alum-precipitate¹⁶.

In the late twentieth century, many advances were made in understanding the mechanisms of the immune system. These included the identification of the major histocompatibility complex (MHC) in mice, antibody production in plasma B cells and validation of the concept of immunological tolerance¹⁷⁻¹⁹. New methods allowed the discovery of the protein structures of antibodies, complement and cytokines. The 1960s saw the beginnings of T-cell biology with the discovery of the involvement of the thymus in cellular immunity by Jacques Miller²⁰. It soon became clear that there were two, interdependent, subsets of lymphocytes²¹. Newly developed reagents, namely monoclonal antibodies, allowed researchers to distinguish and quantify cell surface marker expression. Two mutually exclusive sets of T-cells were identified as either CD4(+) or CD8(+)²². Subsequent discoveries revealed that the two subsets had differing functions; CD4(+) T-cells were found to augment B cells, thus antibody production, while CD8(+) T-cell activity mediated cytotoxicity of infected cells²³⁻²⁵. In 1973, dendritic cells (DCs) were first described by Ralph Steinman and were found to be primary antigen presenting cells (APCs); sampling their environment and presenting foreign proteins to T-cells^{26,27}.

Insights during the 1980's revealed that the roles of CD4 and CD8 receptors were as restriction elements between the two subsets²⁸. CD4(+) T-cells were found to react to antigens presented by major histocompatibility complex (MHC) class II, facilitated by the direct binding of CD4 with MHC II²⁹. Similarly, CD8 T-cells were found to respond to antigens presented by MHC class I³⁰. The interactions of CD4 or CD8 with their corresponding MHC were found to enhance T-cell receptor (TCR) engagement with the MHC-antigen peptide complex³¹⁻³³.

1.2 Molecular basis for the divergent immunological outcomes of protein and DNA vaccination

Lymphocytes are required to distinguish between self and non-self proteins and antigens. Specialised antigen-presenting cells (APCs) capture and display antigens to T lymphocytes, initiating an immune response. These cells also communicate information to T-cells regarding the type of antigen encountered. Up-regulated co-stimulatory and leukocyte adhesion molecules interact with their complementary receptors on the T-cells providing stimulatory or inhibitory signals. The APCs also release cytokines capable of skewing the type of T-cell response initiated³⁴.

Naïve B cells re-circulate between the blood and secondary lymphoid organs, detecting and endocytosing antigens through B cell specific receptors. Antigenic peptides are complexed with MHC-II molecules and presented to CD40L(+) CD4(+) effector T-cells, stimulating antibody production³⁵. Macrophages, resident in most tissues, are actively recruited to sites of inflammation, where they internalise and present antigens on MHC I to CD8(+) effector T-cells, stimulating cell-mediated-immunity³⁶.

Dendritic cells (DCs) and macrophages are professional APCs that can stimulate naïve T-cell differentiation³⁷. APCs can be found in all tissues but upon antigen up-take they migrate to the lymph nodes, where re-circulating T-cell localise. The molecular pathway of antigen presentation is dependent on whether the protein was encountered exogenously to the APC^{38,39}. In general, MHC-II molecules bind peptides arising from the endosomal proteolysis of exogenous proteins via endocytic and phagocytic pathways. MHC-I binds peptides in the endoplasmic reticulum (ER) that are generated from proteasomal proteolysis of endogenous proteins⁴⁰.

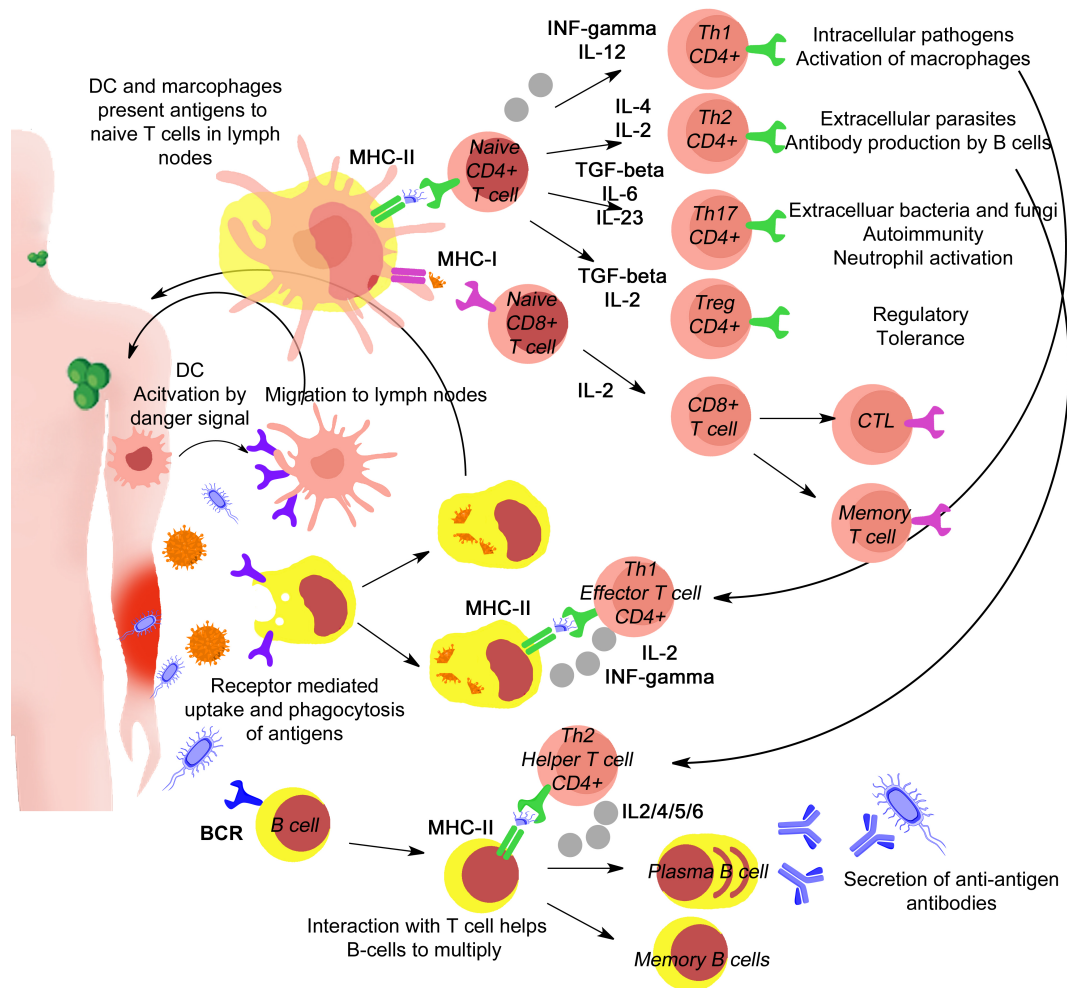


Figure 2 – An overview of professional antigen presenting cells (APCs). Pathogens are internalised by dendritic cells (DCs), macrophages and B cells by receptor mediated endocytosis or phagocytosis. Antigens from the pathogens are then processed and presented on either the major histocompatibility complex (MHC) class-I or class-II (see Figure 3 for more details on this process). DCs and macrophages can migrate to the lymph nodes upon antigen internalisation, where they can present antigens to naïve CD4(+) and CD8(+) T-cells. The DCs and macrophages secrete cytokines that inform the differentiation and proliferation of the naïve CD4(+) T-cells (T-helper type I, 2, 17 or regulatory T-cells), influencing the type of immune response generated against the antigen. Macrophages can also present extracellular antigens, via MHC-II, to antigen specific Th1 effector T-cells at the site of inflammation. This interaction causes the T-cell to secrete pro-inflammatory cytokines, which in turn activate the macrophage. B cells can also present extracellular antigens in a similar manner, however, they present to Th2 helper T-cells, which then produce cytokines causing the B cell to differentiate and secrete anti-antigen antibodies^{37,39}.

This process of molecular distinction between exo and endogenous proteins allows for the direction of the immune response towards either a humoral or cellular response. Exogenous antigen proteins are likely to originate from extracellular pathogens and therefore a strong humoral response is required. By contrast, endogenous peptides are likely to originate from viruses or cancerous cells and therefore require a cellular immune response (**Figure 3**). Such understanding has informed two major strategies for vaccination – DNA- and protein-based vaccines.

Although the major processing routes of exo and endogenous proteins result in MHC-II and MHC-I presentation, respectively, there are exceptions to the rule. CD8(+) T-cell responses specific for exogenous proteins *in vivo* are also common and are an essential component of the host immune response⁴¹. In the mid 1970's Bevan *et al* observed the antigen specific stimulation of MHC-I restricted cytotoxic T lymphocytes (CTLs) after the immunisation of cells that lacked the cognate MHC-I molecule *in vivo*^{42,43}. This and other reports concluded that DCs (and to a limited extent macrophages) have a pathway to present exogenous antigens on MHC-I; this process is termed 'cross-presentation'. DNA vaccines therefore are capable of producing both CD8 and CD4 T-cell responses.

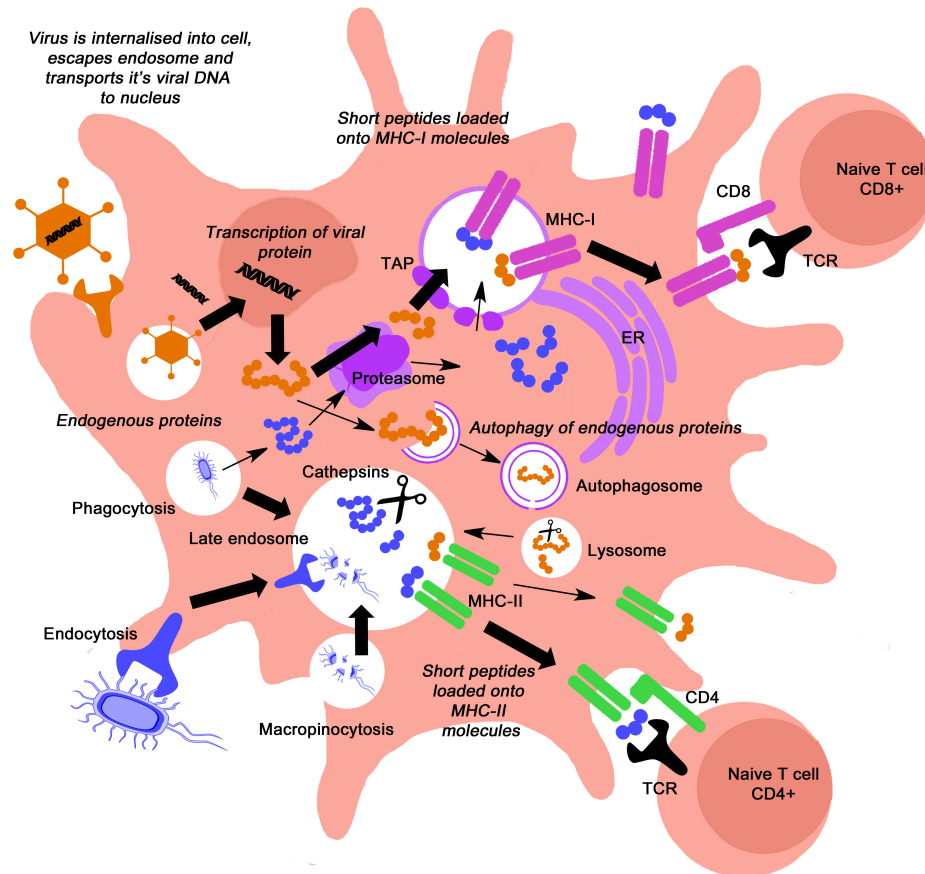


Figure 3 -The mechanisms of antigen processing and presentation by antigen presenting cells (APCs). The major routes are presented by bold arrows. External antigens (blue) are internalised into the APC via receptor-mediated endocytosis, phagocytosis or macropinocytosis and processed in late endosomal compartments. Antigen proteins are then hydrolysed into shorter peptide segments by cathepsins, these peptides are then loaded onto MHC-II molecules. The peptide-MHC-II complexes are trafficked to the cell surface where naïve CD4(+) T-cells can bind the MHC-II with the CD4 receptor, allowing the T-cell receptor (TCR) to interact with the peptide-MHC-II complex. If the affinity of this interaction is high enough, the T-cell will become activated and go on to proliferate and differentiate. Proteins described as endogenous to the APC (orange) can be self-proteins or may originate from viral DNA transcription within the APC. These proteins are processed in the cytoplasm by proteasomes into smaller peptides that are then transported into the endoplasmic reticulum (ER) by a transporter associated with antigen processing (TAP). The peptides are then loaded onto MHC-I molecules for presentation to CD8(+) T-cells. In certain APCs, particularly dendritic cells (DCs), exogenous proteins can be processed via this pathway by retrotranslocation from phagosomes; this is known as ‘cross-priming’. Endogenous proteins can also be trafficked into the late endosome for MHC-II presentation by autophagy^{40,44}.

1.3 DNA vaccines

CD8(+) T-cell mediated cellular immunity has evolved to tackle viral infections, tumors and intracellular parasites. DNA vaccines are best utilized in order to deliver antigen peptides into the MHC-I processing pathway to induce a strong cytotoxic cellular immune response.

Enzo Paoletti and Dennis Panicali reported the first example of a vaccine strategy involving genetic manipulation⁴⁵. The cowpox virus genome was engineered to produce proteins from an influenza virus, eliciting an antibody response against the whole influenza virus in mice. This experiment hailed the beginning of the third generation of vaccines. There are several advantages to DNA vaccination over protein/whole pathogen vaccination. The host's intracellular synthesis of an encoded protein is more likely to result in an antigen that is folded into its native conformation, and is correctly glycosylated and displaying the correct post-translational modifications (PTMs). Recombinant vaccines are often grown in bacteria or yeast, which lack the relevant cellular mechanisms to express mammalian proteins correctly⁴⁶. Protein vaccines suffer from instability issues and some may have a short shelf-life and require cold chain transportation⁴⁷. DNA is relatively more robust and therefore simpler to handle.

In 1990, Jon Wolff found that injection of bacterial DNA plasmids produced an anti-protein response in mice⁴⁸. Three years later Margaret Liu discovered that an intramuscular (IM) injection of DNA, encoding an influenza viral protein, produced protection against influenza in mice⁴⁹. A year later Wang *et al* demonstrated that a plasmid vaccine could induce protective immune responses against HIV-1 antigen expressing cells⁵⁰.

Plasmid transfection is the most simple method of DNA vaccination and has shown some success against herpes simplex virus type 1, malaria, HIV and cancers⁵¹⁻⁵⁴. DNA plasmid vaccines may not be as immunogenic as protein/whole pathogen based vaccines, however, this has been combated by co-administration with chemical adjuvants. An alternative strategy

involves the insertion of sequences encoding for stimulatory cytokines, chemokines, growth factors or even co-stimulatory molecules alongside the antigen in order to bolster, and skew, the immune response⁵⁵⁻⁶¹. A major drawback of the plasmid method is inefficient cell membrane permeability and thus low cellular uptake of the plasmid vectors. High doses of plasmid and multiple boosts are required, especially in larger animals and humans⁶². The nuclear envelope of a eukaryotic cell is a formidable barrier to DNA plasmid vaccination; nuclear pore complexes regulate both passive and active transport across the membrane. Generally, larger molecules must bear nuclear localisation sequences to be actively transported into the nucleus⁶³⁻⁶⁵. It has been estimated that only 1% of DNA, having entered the cytoplasm as a DNA plasmid vector, enters the nucleus⁶⁶. Research has thus focused on improving the efficiency of DNA vaccine delivery. To date, the technologies developed largely fall into two categories, viral and non-viral DNA vectors.

1.4 Non-viral DNA vaccine delivery approaches and vectors

Physical methods of forcing DNA vaccines into cells have several advantages, including the small amounts of DNA required, high transfection efficiency and high immunogenicity. The 'gene gun' uses a blast of pressure to bombard cells with gold microparticles coated with DNA plasmids⁶⁷. The gene gun has been tested on mice and humans however suboptimal transfection and possible tissue damage has been reported⁶⁸⁻⁷⁰. Electroporation is a rapid and efficient method of DNA delivery into mammalian cells and tissues⁷¹⁻⁷⁵. Permeabilization of the cell membrane is achieved through electrical pulses, creating pores that readily seal following vaccination. However, cell membranes can be damaged when high voltages are applied and factors such as intracellular solute concentration and the local charge of surrounding cells can affect the DNA transfection efficiency⁷⁶.

Synthetic, chemical methods of enhancing DNA uptake were first explored in the 1950s. High salt concentrations and polycationic proteins

were used to increase DNA entry into cells⁷⁷. It was found that poliovirus RNA uptake was increased 100 times in the presence of cationic diethylaminoethyl dextran (DEAE-D) and adenovirus (Ad) DNA co-precipitated with calcium phosphate^{78,79}. In 1982, Felgner *et al* developed the first cationic lipid-DNA complex⁸⁰. Since this initial work, DNA has also been complexed with anionic and neutral lipids, demonstrating the versatility of the method⁸¹. Choice of lipid composition can allow for the control of charge, size and the entrapment of other antigens or adjuvants⁸¹⁻⁸³. One of the major limitations of lipid-based vaccine vectors is toxicity upon systemic administration⁸⁴. Another issue relates to the current lack of precise characterisation of the dynamics of lipid-DNA complexes and their cell internalisation and biodistribution *in vivo*⁸⁵.

Alternative polymer-based solutions to the inefficiency of DNA uptake have been developed. Cationic peptide poly-L-lysine (PLL) was found to condense DNA and increase cellular uptake^{86,87}. Polyamidoamine (PAMAN) dendrimers have been used to similar effect⁸⁸. However, polymer based DNA condensates offer little control over the rate of DNA release, relying on acidic pH-induced spontaneous complex dissociation. Researchers have successfully encapsulated DNA into biodegradable microparticles and matrices allowing long-term DNA release without repeat administration^{89,90}. The encapsulation can also offer DNA protection from extracellular DNAses and other immediate causes of degradation upon administration^{91,92}. Chitosan is a bio-degradable, naturally occurring polysaccharide and chitosan nanoparticles have been shown to successfully deliver experimental DNA vaccines, generating effective protection^{93,94}. Liposomes have become a popular method of DNA encapsulation since the first report of the successful vaccination with DNA encoding a hepatitis B antigen, encapsulated in a cationic liposome^{81,95,96}. Liposomes may also exhibit potent adjuvant like properties, depending on their lipid composition⁹⁷.

Such chemical methods of enhancing DNA vaccination generally operate at the first stage of the transfection process; cellular uptake. Even

after successful uptake, DNA must then escape from the endosomal pathways (which lead to degradation), be transported through the cytoplasm and enter the nucleus via nuclear pore complexes. Each of these steps must be addressed by a putative vaccine vector as attrition at each step results in a low percentage of DNA reaching the nucleus^{98,99}. Researchers have developed synthetic approaches to aid at some of these junctions¹⁰⁰⁻¹⁰². The diffusion of DNA through the cytosol also presents challenges; the presence of DNAases has been shown to impede transfection¹⁰³. In one study of nuclear targeting, cationic lipids carrying a DNA fragment were monitored over 24 hours - a maximum of 0.03% of total DNA was ever detected in the nucleus¹⁰⁴. Any potential vector or DNA-delivery complex must also dissociate and release the DNA, the efficiency of which correlates with the efficiency of transfection¹⁰⁵. Trafficking of DNA through the cytoplasm is mediated by nuclear localisation sequences⁶³. Conjugation of these sequences to DNA can facilitate transportation into the cell nucleus¹⁰⁶. These nuclear targeting peptides and some fusion proteins can mediate nuclear transport of DNA¹⁰⁷⁻¹¹⁰.

Viruses have evolved to solve the problems of cellular uptake and DNA transportation. They carry not only effective cell-membrane mediation ligands but also protein machinery for endosomal escape and for the shuttling of nucleic acid into the cell nucleus. Viruses have therefore been considered excellent potential vectors for efficient DNA vaccination and, notably, have been used in the majority of gene therapy clinical trials¹¹¹.

1.5 Viral DNA vaccine vectors

The genetic engineering of viral DNA to produce desirable transgene products was first reported in 1972, when new DNA of therapeutic interest was inserted into the genome of a simian virus¹¹². Vaccinia virus (VV) (a close relation of cowpox) was later altered to the same effect, however initial reports yielded only transient gene expression¹¹³. Viral vectors for

DNA vaccination can be traced to the work of Paoletti *et al*, who altered the cowpox virus genome to produce proteins from an influenza virus, eliciting an antibody response against the whole influenza virus infection in mice⁴⁵. Today, five main classes of viral vectors are considered to be clinically applicable: lentiviruses; adenoviruses (Ad); adeno-associated viruses (AAVs); herpes simplex-1 viruses (HSV-1s) and oncoretroviruses. These classes can be split into two main groups. The first, comprising of oncoretroviruses and lentiviruses, integrate their genome into the host cell chromatin, enabling long-term gene expression and the passage of the transgene to progeny cells. The remaining viruses belong to the second group and insert their DNA into the nucleus where it exists as extra-chromosomal episomes¹¹⁴. Such viral vectors generally achieve high immunogenicity as they carry pathogen-associated molecular patterns (PAMPs); which act specifically as danger signals, stimulating an immune response¹¹⁵. As a result, an additional adjuvant during vaccination is not usually required. The most commonly utilised viral vectors are VV and adenovirus (Ad). In particular, adenovirus type 5 (Ad5) has emerged in recent years as a potentially powerful vector due to its apparent safety and genetic loading capacity^{116,117}.

1.6 Adenovirus (Ad)

Adenovirus (Ad) was first identified in 1953; isolated from human adenoid tissue cultures¹¹⁸. To date, over 50 Ad serotypes have been discovered. Ad is capable of infecting multiple organs, however infection is mostly asymptomatic¹¹⁹. Ad has been used extensively in DNA vaccine research owing to several desirable characteristics: Ad can efficiently deliver DNA directly to the nucleus; has a high capacity for the incorporation of foreign DNA (2-6Kb); is easy to prepare and purify in high titres and has relatively high stability, allowing storage of the virus¹²⁰⁻¹²⁶. However, the use of Ad in the clinic has been limited by broad viral tropism and susceptibility to host neutralisation mechanisms^{123,127}.

Ad5 naturally infects a range of cell types via coxsackie adenovirus receptors (CAR) and heparin sulphate glycoaminoglycans (HSGAGs)¹²⁸⁻¹³¹, which are ubiquitously expressed, resulting in undesired cell tropism. Ad5 is also able to recruit blood coagulation factor X (FX), which acts as a bridging ligand via an interaction with the viral hexon protein, thus causing additional hepatocyte infection and hepatic toxicity¹³²⁻¹³⁶. Moreover, Ad5 bound by complement is readily sequestered by erythrocytes and is subsequently rapidly cleared from host circulation¹³⁷. As Ad5 is prevalent in the environment, up to 70% of the population carry anti-Ad5 antibodies^{138,139}. These antibodies efficiently neutralise Ad5, rendering it unable to infect target cells and unable to deliver the intended DNA cargo. Together, these issues represent the major obstacles to Ad-mediated gene transfer in a therapeutic setting.

Several approaches have attempted to circumvent individual aspects of these problems, including Ad pseudotyping, genetic or polymeric modifications of viral capsid proteins, the creation of chimeric Ads, the conjugation of viral vectors with antibody or fusion proteins and the use of rare or non-human serotypes of Ad¹⁴⁰⁻¹⁵⁰. Pseudotyping involves the exchange of the viral vector cell attachment proteins with proteins from another, related virus, which displays the desired tropism. Ad, retroviral and lentiviral vectors have been modified with pseudotyping¹⁵¹. Pseudotyping, however, restricts the vector to a subset of pre-existing tropisms while the introduction of fusion proteins can negatively impact on production yield and requires extensive genetic manipulation.

Adaptor proteins offer a great deal of flexibility and the same viral vector can be coupled with multiple targeting proteins. Ad has been targeted to DCs via fusion with CD40L^{152,153}. Antibodies have also been incorporated into viral vectors and used as targeting ligands^{144,154}. Advantageously, adaptor proteins do not require direct changes to the virus that could result in problems in vector production and may interfere with transfection efficiency. However, there are issues facing two component

systems, primarily the stability of the complex and the heterogeneity resulting from any subsequent dissociation.

Peptides and proteins have been genetically incorporated into Ad viral vectors; this approach has proven to be very versatile and yields a single-component system, favoured for clinical application. The successful alteration of tropism has been achieved through the incorporation of single point mutations, small targeting motifs (such as an Arg-Gly-Asp (RDG) peptide), single-chain antibodies, growth factors and attachment proteins¹⁵⁵⁻¹⁶². Genetic incorporation allows great scope for cellular targeting, however, some strategies require genetic manipulation of the virus that can be technically challenging. With larger proteins, problems during viral replication can occur. The transgenic protein may not naturally be synthesized within cells in the same manner or location as the virus and therefore the processes of protein folding, disulphide bridge formation and post-translational modification can be impeded^{163,164}.

The chemical modification of viral vectors could offer a simpler modification technique with both a broad scope for targeting and high versatility. Chemical methods also allow the introduction of ligands that cannot be genetically incorporated such as saccharides and lipids, in addition to unnatural potential binding agents. Polyethylene glycol (PEG) is a non-immunogenic and nontoxic material that has been used to increase the plasma half-life of therapeutic proteins^{165,166}. The PEGylation of Ad has been studied extensively¹⁶⁷⁻¹⁷⁷ and whilst PEG modification alone only served to narrow the Ad tropism, PEG conjugated to targeting ligands also offered retargeting to desired cell types^{168,178,179}. Poly-N-(2-hydroxypropyl) methacrylamide (poly-HPMA) and polysaccharides have also been grafted onto Ad through reaction with multiple amino acids on the capsid surface^{180,181}. Grafting polymers onto a viral capsid surface could impede the viral uncoating and endosomal escape required for DNA transduction. Although the data published for the grafted Ads demonstrate transduction is possible, a direct comparison between the efficiency of transduction between coated and non-coated has not been drawn¹⁴³.

1.7 DNA vaccine vector targeting: DC or not DC?

Targeting to a specific cell-type is an accepted strategy for enhancing DNA vaccination and other forms of gene-therapy (GT)¹⁸². The native tropism of viral vectors is often undesirable in a therapeutic context and can cause toxic side effects. For instance, Ad readily infects hepaocytes and can result in liver toxicity¹³⁵. Equally, viruses may have little or no ability to infect the cell-type required for successful GT or vaccination¹⁸³. Another consideration is that broad natural tropism may result in the rapid clearance of the virus from the host, thus reducing the amount of transgene to reach the site of therapeutic interest¹⁸⁴.

DCs are considered the principal APC; they reside in peripheral tissues as sentinels, sampling their environment for signs of infection. Alerted to the presence of a pathogen, via the interaction of PAMPs with the DC's Toll Like Receptors (TLRs), the DCs capture the antigen and migrate to the nearest lymph-node. After processing the antigen into peptides, the DCs present their findings to CD4(+) or CD(8)+ T-cells via MHC II or I, respectively. As described previously, the designation of the antigen to MHC I or MHC II is largely dependent on whether the antigen originated from the extracellular environment or from inside the DC³⁹. DCs also communicate information to T-cells regarding the type of antigen collected and whether a tolerogenic or immunogenic response is required. This is achieved through the release of cytokines and the up-regulation of co-stimulatory molecules, which bind to their respective ligands on the T-cells¹⁸⁵. The T-cells proliferate and differentiate, the phenotype that results is dictated by the signals received from the DCs. DCs can also aid in antibody production by direct antigen presentation to B cells¹⁸⁶⁻¹⁸⁸. Immune tolerance is also mediated by DCs, through the stimulation of regulatory T-cells (Tregs) and by T-cell deletion¹⁸⁹. These central roles of DCs in both generating and regulating immune responses, are a powerful incentive for the successful development of a DC-targeted DNA vaccine^{190,191}.

The ligand utilised to target the chosen DC subset may also act as an adjuvant; ensuring the simultaneous delivery of adjuvant and antigen. The targeting of an antigen to dectin-1 (an immune stimulation receptor) with an antigen-antibody conjugate elicited a potent CD8(+) T-cell response *in vitro*¹⁹². Targeting vaccines to DCs therefore offers not only enhanced potency of vaccination but also the opportunity to tailor the type of immunity induced via the selection of target receptor and conjugated adjuvant molecule^{193,194}.

1.8 Lectins as targets

Carbohydrates are ubiquitous *in vivo* and are crucial to many biological processes, including cell-cell and cell-microbe interactions. These interactions are mediated by lectins displayed on cell surfaces, which bind to specific sugar moieties. Many immune cells, including DCs, express a number of lectins important for pathogen recognition, cellular interaction and signalling. The lectins identified on DCs include the mannose receptor (MR), dendritic cell immunoreceptor (DCIR), sialic acid-binding immunoglobulin-type lectins (SIGLECs), dectin-1 and 2, DEC-205, macrophage galactose lectin (MGL), langerin and DC-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN)¹⁹⁵⁻²⁰³. All of these lectins have demonstrated some degree of sugar specificity and immunomodulation potential (**Table 2**).

Table 2 – The lectins expressed by dendritic cells (DCs). Known ligands of the lectins and any reported immune modulation capabilities are detailed. Abbreviations; tyrosine-based activation motif (ITAM); tyrosine-based inhibitory motif (ITIM); No Data (ND); toll-like-receptor (TLR); sulfonated-Lewis –X (sLe-X); dendritic cell-associated C-type lectin (Dectin); Macrophage- inducible C-type lectin (Mincle); C-type lectin (CLEC); dendritic cell immunoreceptor (DCIR); Blood dendritic cell antigen-2 (BDCA-2); Mannose receptor (MR); Sialic acid-binding immunoglobulin-type lectins (SIGLEC); Dendritic cell natural killer lectin group receptor 1 (DNGR-1); Dendritic cell asialoglycoprotein receptor (DC-ASGPR); lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1); Myeloid inhibitory C-type lectin-like receptor (MICAL); F4/80-like receptor (FIRE); Dendritic cell-specific transmembrane protein (DC-STAMP)²⁰⁴⁻²⁰⁹.

Receptor	Ligand	Immune modulation?	Notes
DEC-205	CpG ²¹⁰	Antigens targeted to DEC-205 in mice presented in tolerogenic way ^{211,212} .	Also expressed by thymic epithelial cells. Receptor for dying cells, potential tolerance ²¹³ .
MR	Branch-end mannose, fucose, GlcNAc, sLeX ²¹⁴ (rev.Taylor2005) Lactoferrin ^{215,216} .	Increase IL-10 and IL-1Ra Reduced TLR stimulated IL-12 ²¹⁷ .	No cytoplasmic tail
DCIR	ND	Inhibits TLR8 mediated cytokine production ^{218 219} .	Cytoplasmic ITIM motif
SIGLEC	Sialic acids ^{197,220}	Inhibition of several proinflammatory responses including TLR signals	SIGLEC9 present on DCs Cytoplasmic ITIM or ITAM motifs
Dectin-1	β -1-3- glucans ^{198,221}	Proinflammatory ^{222 223 224} , Enhances TLR-2 responses ²²⁵ , Increases TLR mediated TNF and IL-12, Regulatory response to TLR-2 stimulus ²²⁷	Phagocytosis Anti fungal immunity and induction of Th17 Cytoplasmic ITAM motif direct signalling via tyrosine kinase ^{228 229}
Dectin-2	High mannose α -mannans ^{230,231}	Proinflammatory IL-4 increase UV-Induced tolerance to contact hypersensitivity ²³²	Similar to dectin-1 but also anti-bacterial/fungal immunity and allergy ²³³

Langerin	Mannose, GlcNAc, Fucose, s6SLeX ^{234,235}	Possible involvement in activation IFN- γ CD8(+) T- cells ²³⁶	Not only present on langerhan cells but other DC subsets
BCDA- 2/CD303/CL EC4C/ DLEC	Asialo-galactosyl- oligosaccharides ²³⁷	Reduced TLR induced type I interferins and IL-12 ²³⁸	Expressed by pDCs. Cross- linking inhibition of IFN-I production ²³⁹
CLEC-1	ND	Possible enhanced regulatory mediation and moderation of Th17 differentiation in myeloid and endothelial cells ²⁴⁰	PBMC derived DCs ²⁴¹ Does not have ITAM or inhibitory motifs
DNGR- 1/CLEC9A	Actin filaments	Binds dead cells DAMPs ^{242,243}	Sense nonhomeostatic cell death
DC-ASGPR	Asialoglycoprotein	Generates IL-10 producing CD4(+) T-cells ²⁴⁴	Immunoreceptor tyrosine based activation motiflike ²⁴⁵
DC-SIGN	Internal mannose in high mannose ^{204,246} polysaccharides Lactoferrin ²¹⁵ Terminal fucose bearing saccharides Lewis blood antigens ²⁴⁷	Fucose binding causes increased IL-10, anti- inflammatory (IL-12 decrease) response Modulation of TLR 4 ^{248,249}	Lactoferrn combats excessive inflammation Mediates interactions with DCs and neutrophils ²⁵⁰
MINCLE	α -mannose Trehalose-6'6'- dimycolate ²⁵¹ Damage-associated molecular patterns (DAMPs)	Interacts with Fc receptor common γ -chain. NK-kB activation ²⁵²	Fungi and necrotic cells ²⁵³ Senses non- homeostatic cell death ²⁵⁴
LOX-1	Oxidized low-density lipoproteins ²⁵⁵	Signalling on DCs promotes B cell differentiation ²⁵⁶ .	Lectin like. Expressed on DCs , B cells macrophages and smooth muscle
MGL	Terminal GalNAc	Binding CD45 to inhibit T- cells ^{257,258}	Expressed on immature DCs
MICL/CLEC 12A	Monosodium urate crystals released from dead cells ²⁵⁹	Inhibitory for dead, self cells.	Low expression on monocytes, macrophages and B cells. High expression on CD8(+) and pDCs ITIM in cytoplasmic tails

			260
CLEC12B	ND	Inhibition of positive ITAM-derived signals ²⁶¹	
FIRE	ND	Induced humoral immunity in the absence of conventional danger signals ²⁶²	Expressed on CD8(-) immature DCs
DC-STAMP	ND	Cell-cell fusion during osteoclastogenesis ^{263,264}	DCs and activated blood DCs ^{265,266} ITIM in the cytoplasmic tails

Several immune cell types express many of these lectins, however, the expression of DC-SIGN is restricted to DCs and a small population of other APCs. DC-SIGN is expressed on monocyte and CD34(+) derived DCs and dermal DCs. However, immature DC in peripheral tissues, lymphoid tissues, tonsils and spleen express the highest levels²⁶⁷. DC-SIGN has also been found on two DC precursor populations and on decidual macrophages, Hofbauer cells and alveolar macrophage²⁶⁷⁻²⁷⁰. Where it supports primary immune responses through pathogen recognition and internalisation, and by processing antigens to intracellular compartments²⁶⁷. DC-SIGN is a calcium-dependent, type II transmembrane protein containing a short cytoplasmic N-terminal tail, an extracellular domain consisting of seven repeating units followed by a C terminal carbohydrate-recognition domain (CRD)^{267,271}. DC-SIGN recognizes high-mannose structures, binding predominantly to internal mannose residues in the polysaccharide structure^{204,272,273}. The lectin can also bind to the fucose containing Lewis blood-group antigens, Lewis-A, B, Y and X²⁴⁶ (**see chapt. 2.1, figure 1**). Lewis X has been highlighted as an efficient and specific DC-SIGN ligand^{274,275}.

Lectin targeting can be achieved through the corresponding sugar or an anti-lectin antibody. The latter strategy has been implemented in the targeting of protein vaccines to DC-SIGN²⁷⁶⁻²⁷⁸. Hesse *et al* used an OVA-anti-DC-SIGN conjugate to target their vaccine to DCs and demonstrated an increase in anti-OVA CD8(+) T-cell immunity²⁷⁸. Nanoparticles and liposomes, coated with anti-DC-SIGN antibody and loaded with antigen,

have also been targeted to DCs; yielding 10-100 fold increases in antigen-dependent T-cell responses^{279,280}.

Singh *et al* were the first to use a sugar-protein conjugate in order to target a vaccine protein to DC-SIGN. The Lewis B and X saccharides are ligands for DC-SIGN. These carbohydrates were oxidised and functionalised with a maleimide group, which was then conjugated to free cysteines of OVA. Vaccination with targeted OVA-carbohydrate conjugates in humanised, transgenic DC-SIGN mice, resulted in over 5 times greater antigen specific T-cell proliferation²⁷⁴. Poly(amido amine) dendrimers have been modified with the DC-SIGN ligand Le-B and were used to successfully deliver protein antigens to DCs²⁸¹. Similarly Le-B and Le-X modified liposomes have been shown to target DC-SIGN²⁸².

Glycan subunits and structure can modulate lectin function. Binding of either mannose- or fucose-based sugars to DC-SIGN can modulate immune responses to TLR-4 stimulation²⁸³. Geijtenbeek *et al* have shown that stimulating DC-SIGN, through the use of carbohydrate ligands, can induce skewing of helper T-cell responses^{248,283}. Carbohydrate immune modulation is an emerging field; discoveries are indentifying specific carbohydrate structures able to activate DCs to elicit particular T-cell responses²⁸⁴. Using sugar-vaccine conjugates to target DCs via lectins could potentially allow tuning towards a more designed, therapeutic immune response.

A study was conducted comparing antibodies and carbohydrate to target vaccines to DC-SIGN²⁸⁵. Nanoparticles (NPs) encapsulating MHC-I or MHC-II restricted antigens were coated with anti-DC-SIGN antibodies, HIV gp120 glycans, the Le-X trisaccharide or ManLAM. The authors concluded that the anti-DC-SIGN antibody coated NPs were around 2-fold more effective at DC-specific internalisation. However, the same researchers later reported that the divergent binding modes of antibody and carbohydrate ligands could modulate receptor trafficking^{277,286}. Antibody-bound DC-SIGN molecules remained inside cells for prolonged periods, resulting in reduced receptor expression levels. In addition to this

effect, the resurfaced DC-SIGN molecules showed a reduced ability to internalise. These effects were not observed with the carbohydrate ligand targeting²⁸⁵. Indeed, glycosylated protein ligands for DC-SIGN are internalised at greater levels than the estimated receptor expression should allow. This suggests that DC-SIGN recycles to the cell surface following the release of carbohydrate ligands within the endosome²⁸⁷. The use of anti-DC-SIGN antibodies, although arguably the most robust method in terms of guaranteeing target specificity, may thus not be optimal for vaccine delivery. Care must be taken to ensure the correct part of the lectin structure is used to generate the antibody. Antibodies are relatively large structures, compared to even the most complex polysaccharide and, as such, loading onto viral vectors may present steric problems; blocking viral uncoating and endosomal mechanisms. Generally, fewer antibodies can be loaded onto a vector, compared to small molecule carbohydrate ligands²⁸⁵.

1.9 Targeting Adenovirus (Ad) to dendritic cells (DCs)

Ad, as a promising viral DNA vaccine vector, has been modified to target DCs. Ad5 does not efficiently infect and transduce human monocyte derived DCs *in vitro*²⁸⁸; modified Ads attempt to improve DC infection through targeting DC receptors. Amino acid mutations of the Ad capsid, inserting additional RDG integrin binding motifs, have improved DCs transduction^{289,290}. Bispecific anti-Ad, anti-CD40 antibodies have been used to target Ad vectors to mouse DCs *in vivo*. These studies chose a tumour model antigen encoding Ad to modify and achieved enhanced CD8(+) T-cell anti-tumour protection compared to vaccination with the unmodified Ad^{291,292}. The CD40 receptor has also been successfully targeted through the covalent linkage of a novel CD40-binding peptide to the Ad capsid²⁹³. A genetically modified Ad, able to be conjugated with anti-DC-SIGN antibody has been shown to effectively target DCs and induce IL-12 production²⁹⁴. Similarly, a genetically modified Ad vector containing a biotin acceptor peptide at the C terminus of a capsid protein has been developed. The Ad is then able to be metabolically biotinylated and bound to any ligand via a

biotin-avidin bridge²⁹⁵. Maguire *et al* used this method to introduce three different DC targeting binding ligands to the Ad surface. The $\alpha_v\beta_3$ integrin, ChemR23 and DC-SIGN receptors have also been targeted using high affinity binding peptides, or monoclonal antibodies.

Targeting to DC-SIGN proved the most effective strategy and resulted in the highest levels of DC transduction compared to other endocytic receptors²⁹⁶. There are several limitations in targeting DC-SIGN via the aforementioned methods; firstly genetic modification of the Ad capsid can be laborious and may affect the stability of the vector. Secondly, the modifications are not covalently attached; therefore dissociation is possible over time and in high protein concentration environments, such as serum. Thirdly, using targeting antibodies restricts targets to those for which high avidity antibodies have been developed. A final point to note is that no studies have yet been conducted as to whether the location and size of these modifications inhibit the viral endosomal escape and transduction mechanisms.

Ad targeting to DCs via DC-SIGN could produce a more effective DNA vaccination. The restriction of DC-SIGN to APCs, principally DCs, and the high specificity of the receptor for high mannose and Lewis X saccharide structures, makes DC-SIGN an attractive target. Carbohydrates, despite generally having lowered specificity relative to antibodies, are easier to produce in large homogenous batches. Structural and chemical specificity can be introduced during the synthesis, allowing for the exploration of sugar immune modulation. The focus of this thesis was to utilise sugar ligands to target Ad DNA vaccine vectors DCs. Mannose or Lewis-X carbohydrates were covalently bound to the Ad capsid surface, generating new 'glyco-Ads'. The sugar coating was able to alter the Ad tropism, to infect exclusively DC-SIGN(+) DCs and offer the Ad protection from host neutralisation and sequestering mechanisms. Furthermore, these targeted glyco-Ads are shown to be better able to transduce DCs *in vitro* and induce more CD8 T cell proliferation. *In vivo*, the DC-SIGN targeting of the glyco-Ads produced more potent CD8 T cells in vaccination studies.

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- 294 Korokhov, N. *et al.* High efficiency transduction of dendritic cells by adenoviral vectors targeted to DC-SIGN. *Cancer Biology & Therapy* **4**, 289-294 (2005).
- 295 Parrott, M. B. *et al.* Metabolically biotinylated adenovirus for cell targeting, ligand screening, and vector purification. *Mol Ther* **8**, 688-700 (2003).
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2. Imidate sugar synthesis

2.1 Introduction

2.1.1 Lectins and sugar binding

Carbohydrates are ubiquitous in nature, and are key to many biological functions. Glycolipids and glycoproteins are found on the surfaces of mammalian, bacterial and fungal cells. The specific, reversible interactions between saccharide structures and their binding receptors (lectins) are pivotal to both cell-cell and cell-microbe interactions. The binding of lectins to their individual sugar targets often has a low affinity, however this is compensated for through the presence of multiple binding events¹. Subsequently, the entirety of the interaction is greater than the sum of its individual binding parts, a concept termed multivalent binding¹.

Lectins often contain more than one carbohydrate-binding site per molecule. Concurrently, sugars are generally presented in clusters or polymers^{1,2}. Researchers attempting to build artificial lectin-targeting constructs must utilize multivalency in order to achieve optimal binding. Different types of multivalent scaffolds have been used to selectively bind lectin-specific carbohydrates, including carrier proteins, dendrimers, micelles, nanoparticles and polymers³⁻¹⁰.

Lectins exhibit diverse methods of multivalent binding and can display carbohydrate recognitions domains (CRDs) in both planar and three-dimensional space². Dendritic cell (DC)-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) is a type II calcium-dependent (C-type) lectin that is involved in binding to both host glycoproteins and invading microorganisms. DC-SIGN is primarily expressed by immature dendritic cells (DCs) in peripheral tissue and mature DCs in the lymphoid tissues¹¹. Expression has been reported on some subgroups of macrophages, however it has been established that DC-

SIGN expression correlates with a cell's potential to present antigens¹²⁻¹⁵. Many examples of targeting vaccines to DCs using DC-SIGN have yielded greater levels of antigen presentation than untargeted vaccines, and preclinical data has demonstrated promising vaccination results¹⁶⁻²⁰.

The DC-SIGN molecule consists of a cytoplasmic tail and transmembrane region followed by an α -helix stalk, capped by a single CRD. The stalk region interacts with the corresponding stalks of three other DC-SIGN molecules to form a tetramer. This association projects the CRDs 20-30nm from the cell membrane, with a 4-6nm space between each CRD²¹. The DC-SIGN tetramers cluster in groups of up to 20, forming nano-domains on the cell surface²²⁻²⁴. The tetramerisation and clustering of DC-SIGN enables the multivalent binding platform required to overcome the low affinity of the single carbohydrate-lectin interaction^{25,26}.

In order to target DC-SIGN, multivalent interactions between the artificial carbohydrate ligand and the lectin must be designed/employed²⁷⁻³¹. Single amino acid mutation experiments have demonstrated that the CRD of DC-SIGN is capable of binding different saccharides³². The lectin binds mannose monomers within high mannose polymers and fucosylated glycans, including the blood-type Lewis antigens, LeX, LeB, LeX, LeY and sulfo-LeA²⁶ (**Figure 1**). Upon recognition of these ligands, DC-SIGN is internalised via the endo-lysosomal pathway, resulting in either antigen presentation on MHC-II or cross presentation on MHC-I^{33,34}. Cross presentation can be enhanced by simultaneous triggering of toll-like receptors (TLRs)³⁵.

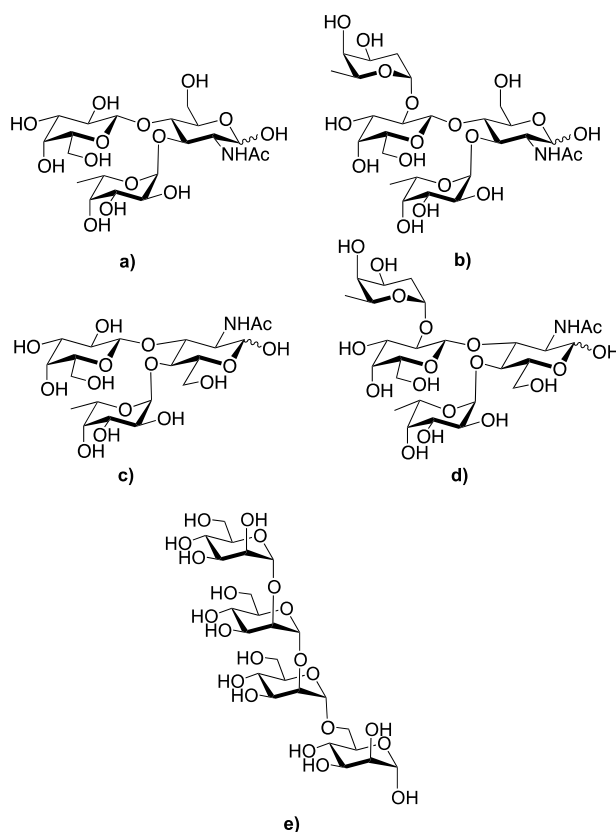


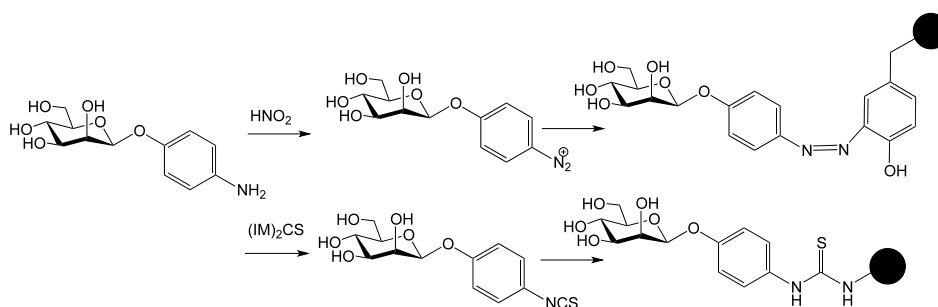
Figure 1 - The Lewis blood antigen structures a) Lewis-X b) Lewis-Y, c) Lewis-A, d) Lewis-B, e) α -1,2-mannose, an example of a high mannose structure found on pathogens²⁷.

The LeX trisaccharide is the most specific ligand for DC-SIGN³⁶. Conversely, LeY, LeA, LeB and sulfonated LeX have shown specificity for L-SIGN, which is not an antigen presenting cell (APC)-exclusive lectin²⁸. The DC immunoreceptor (DCIR) is the only other human DC-specific receptor that has been reported to interact with LeX, however the reported binding affinity is much lower than that of DC-SIGN³⁷. Carbohydrates such as LeX could therefore be used to selectively target adenovirus (Ad) vaccine vectors to DCs. Alternatively, mannose-based sugar ligands could also be used. However, mannose receptors are more broadly expressed, which would limit selectivity. To investigate the potential of using sugar-lectin systems to target DC-SIGN, both mannose and LeX sugars were synthesised with a biocompatible linker. These were then used to coat the Ad capsid for delivery of DNA vaccines to DCs.

2.1.2 Methods of carbohydrate-protein conjugation

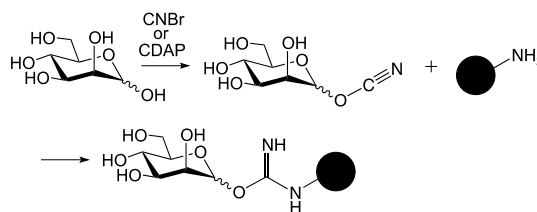
Many chemical methods of conjugating small molecules, saccharides and peptides to proteins have been explored³⁸⁻⁴⁰. The chemical reactivity of protein scaffolds generally depends on the side chains of the amino acids, or the *N*- and *C*- terminal residues^{38,39,41}. Bioconjugation methods that do not require prior protein functionalisation and allow a single point attachment of the carbohydrate to existing amino acids would be ideal for the chemical glycosylation of Ad.

In 1929, the first conjugation of glycosidic aromatic diazonium salt electrophiles to proteins was reported (**Scheme 1**)⁴². These sugars reacted with several amino acid R-groups, including tyrosine, tryptophan, lysine and histidine⁴³.



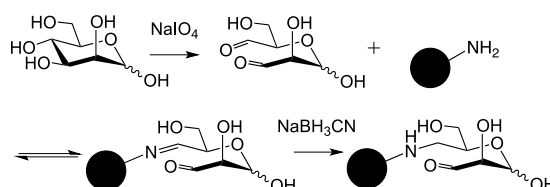
Scheme 1 - Aromatic diazonium salts, derived from the corresponding *p*-aminoaryl glycosides. Phenylisothiocyanates react more readily with primary amines⁴⁴.

Cyanylation with cyanogen bromide (CNBr) has been used to produce saccharide-protein conjugates (**Scheme 2**)⁴⁵. CNBr is toxic, although less hazardous cyano complexes have been developed^{46,47}. Additionally, high equivalents must be employed as most of the reagent hydrolyzes in aqueous solutions at biocompatible pH values⁴⁸.



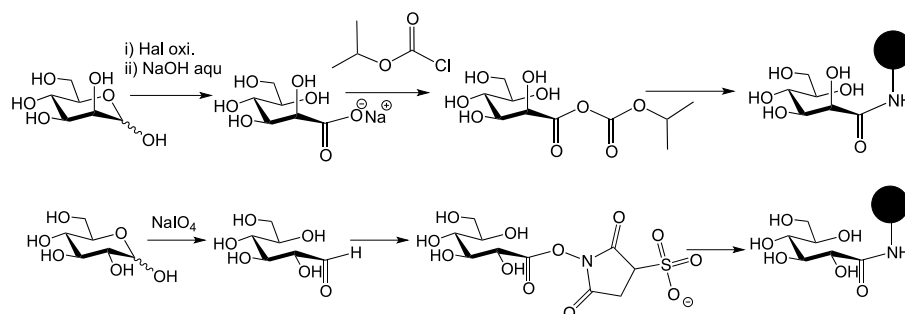
Scheme 2 – Cyanylation of sugars for reaction with protein amino groups

Another grafting method for conjugating polysaccharides to proteins involves the oxidation of saccharide diols with periodate, Dess-Martin periodinane or TEMPO, yielding reactive aldehydes or ketones (**Scheme 3**). These groups react reversibly with amines to form imines (Schiff bases) that undergo reductive amination, forming stable, secondary amines⁴⁹⁻⁵².



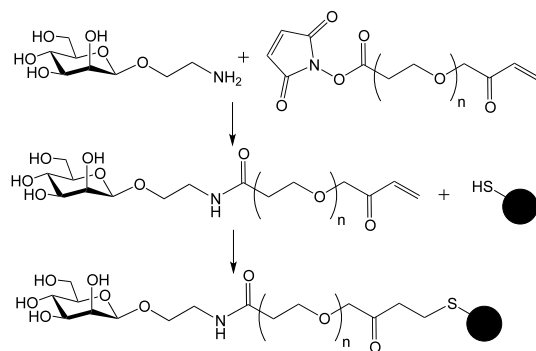
Scheme 3 - Polysaccharides can be oxidised to form reactive aldehydes, which react reversibly with primary amines. The subsequent Schiff base formed can then be trapped by reduction.

Amide bonds are very stable under biocompatible conditions and have been utilised extensively for bioconjugation^{53,54}. Ashwell *et al* described the formation of 'sugar acids' through bromine oxidation. The subsequent aldonic acid can react with nucleophilic primary amines on the protein (**Scheme 4**)⁵⁵. Glycosylamines have been synthesised to conjugate to protein carboxyl groups, via reaction with coupling agents^{56,57}. Carbodiimide based activation of carboxyl groups can be used to make 'zero length' amide cross linkers, through the addition of an auxiliary nucleophile, usually 3-sulfo-1-hydroxysuccinimide (S-NHS) or 1-hydroxy-1,2,3-benzotriazole (HOBt)⁵⁸.

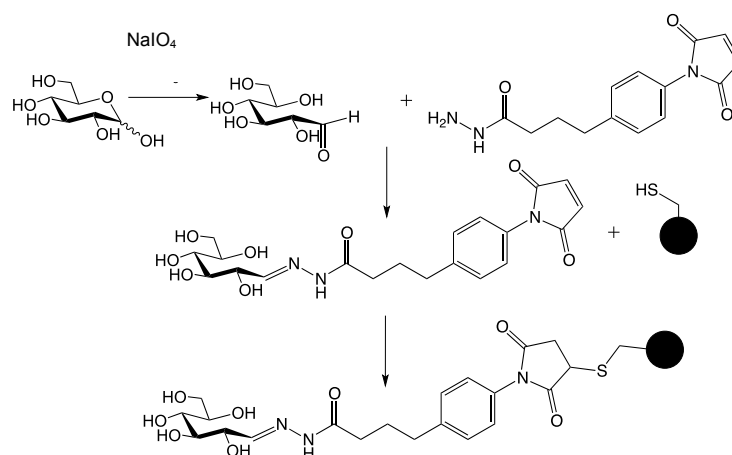


Scheme 4 - Aldonic acid formation, via halogen oxidation, activation and subsequent reaction with primary amine on protein to form an amide bond.

Linker groups offer the opportunity to define the point of protein attachment to the saccharide. Stable thioester bonds can be formed via the reaction of *N*-hydroxysuccinimide ester (NHS-ester)-functionalised saccharides and modified thiol groups on proteins. Similarly, modified thiol groups can react with halocarbonyls⁵⁹. Complex carbohydrates, bearing an acrylate linker based on a triethylene glycol moiety, have been conjugated to antigenic peptides via thiol groups (**Scheme 5 and 6**). Sugars are also commonly oxidised to introduce linker groups, through reaction with hydrazine for example. This method, as well as photochemically or thermal induced radical additions of thiols to alkenes, have been used to generate polysaccharide-based vaccines⁶⁰. Singh *et al* conjugated oxidised LeX and LeB to OVA protein to generate DC-SIGN-targeting vaccines (**Scheme 6**)⁶¹. Free thiol moieties are not common on proteins, which improves region-selectivity but limits opportunities for carbohydrate conjugation. Halocarbonyls, NHS esters and additional thiols can be chemically introduced into proteins, however this requires additional steps and purifications that may denature the Ad capsid³⁸.

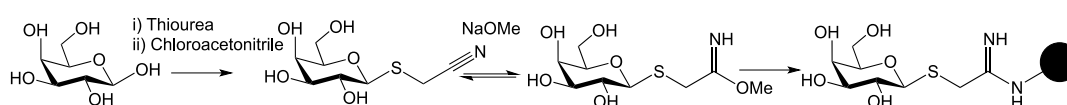


Scheme 5 - Carbohydrates are modified with an acrylate linker, based on a triethylene glycol moiety, have been conjugated to proteins via thiol radical chemistry⁵⁹.



Scheme 6 - Stable thioester bonds can be formed via the reaction of NHS-ester functionalised saccharides and modified thiol groups on proteins.

The use of 2-imino-2-methoxyethyl-1-thioglycosides (IME) functionalised carbohydrates for the conjugation to proteins was first described by Lee *et al* in 1976⁶². Galactose, glucose and mannose were functionalised with a cyanomethyl thiol group at the anomeric position (**Scheme 7**). Subsequently, the cyanomethyl thiol group was converted to the amine-reactive IME group, through nucleophilic reaction with sodium methoxide. The IME sugars reacted readily with simple amines, amino acids and whole proteins. Previous work has successfully demonstrated the conjugation of monosaccharide IME sugars with the capsid proteins of Ad⁶³.



Scheme 7 - Carbohydrates modified with cyanomethyl thiol can be activated to the primary amine reactive IME group. The reaction with protein lysine residues yields an imidamide bond.

The alteration of Ad tropism possible is dependent on the complete abrogation of the natural infection routes. It was hypothesised that a significant percentage of the total capsid would need to be modified with carbohydrates, in order to block the native receptor binding sites. Espenlaub *et al* reported that a total of 60% capsid coverage resulted in a

100-fold decrease in CAR-dependent infection⁶⁴. Sugar modifications must also perform as targeting ligands towards a lectin of choice. In this system, thiol-reactive conjugation methods would not offer enough modification density per Ad in order to achieve these goals. The use of aryl functional groups may result in non-specific hydrophobic interaction with hydrophobic patches on the Ad capsid and/or other proteins *in vivo*. Carboxylic acid residues on Ad could also be used for conjugation, however this would result in a large change to the overall isoelectric point (pI) of the virus. The surface charge of a virus can impact on its capsid stability and also alter biodistribution and tropism⁶⁵⁻⁶⁷. Reductive amination has been used for the poly-mannosylation of the Ad capsid⁶⁴. However, oxidation of polysaccharides may destroy lectin recognition and therefore this would not be an appropriate method for our targeting proposes. Indeed, Espenlaub *et al* observed that the mannosylation of Ads, via reductive amination, decreased the transduction of DCs, despite DCs expressing mannose receptors (MR). Cyanylation of a mono or polysaccharide could have a similar effect as the position and number of cyanylations are not controlled⁴⁵. Therefore, it is probable that several polysaccharide chains are grafted to the protein, rather than a single point attachment.

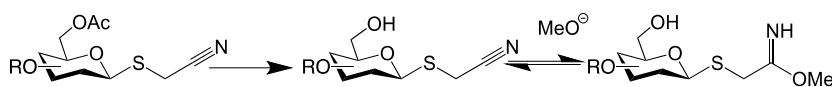
Lysine residue conjugation offers the potential to attach many copies of a sugar to the Ad capsid (22,00 lysine residues/virus⁶⁸). Commonly, either NHS-esters or isothiocyanate have been employed to conjugate small molecules with lysine. The resulting amide and thiourea bonds alter the pI of the virus, which can induce structural changes or alter solubility and tropism⁶⁹⁻⁷¹. The reaction of 2-imino-2-methoxyethyl-1-thioglycosides with lysines forms an imidamide bond and maintains the native pI of proteins⁷¹. Advantageously, the cyanomethyl thiol precursor to the IME group can be installed early in an oligomer synthesis, since it is orthogonal to all subsequent chemistry. This further allows for the derivatization of other desirable oligosaccharide structures further along the synthetic route⁷¹. Thus, we envisioned the production of various IME

sugars that we could use to chemoselectively functionalize Ad capsid proteins.

2.2 Results

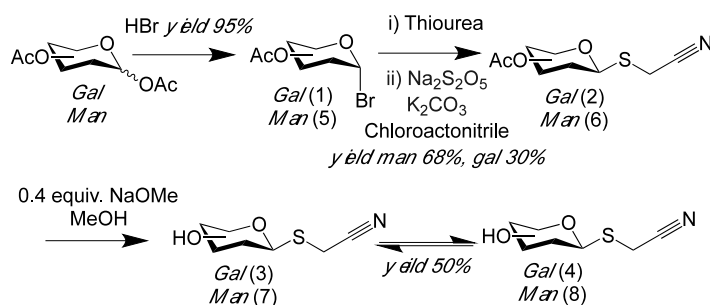
2.2.1 Synthesis strategy

Mannose (Man) IME and LeX IME were synthesised to target MR and DC-SIGN, respectively. IME Galactose (Gal) was synthesised as a non-specific control. Following their synthesis, the acylated cyanomethyl thioglycosides were purified and stored under an inert atmosphere until used for protein modification. The activation and deacylation of the thioglycosides to the lysine reactive IME was achieved by catalytic amounts of NaOMe in MeOH (Scheme 8). Equilibrium was established between the cyanomethyl and 2-imino-2-methoxyethyl-forms of the sugars.



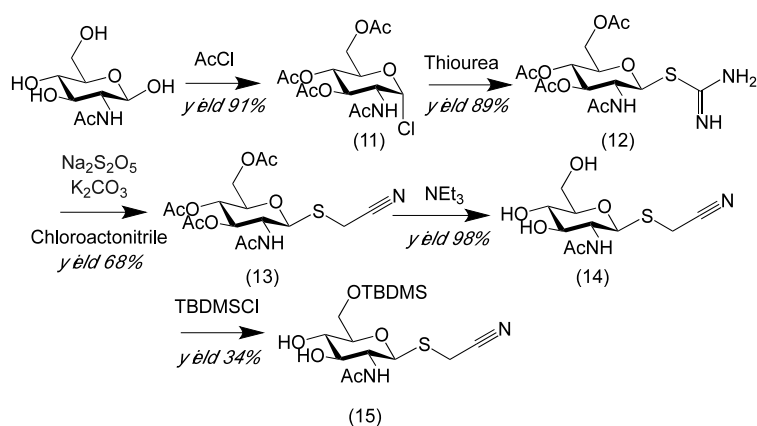
Scheme 8 – The simultaneous deacylation and activation of a generalized cyanomethyl thioglycoside 2-imino-2-methoxyethyl-1-thioglycoside (IME).

Gal- and Man- IME sugars were synthesised in 4 steps (Scheme 9). The overall yield was 32% for IME-mannose and 14% for IME-galactose.

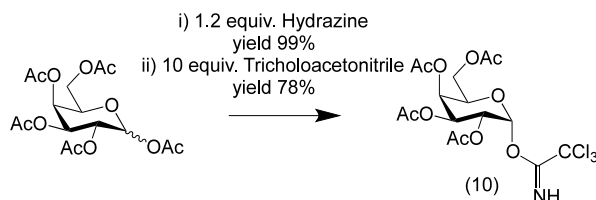


Scheme 9 – Synthesis of mannose- and galactose-based 2-imino-2-methoxyethyl-1-thioglycoside (IME) sugars (4) (8).

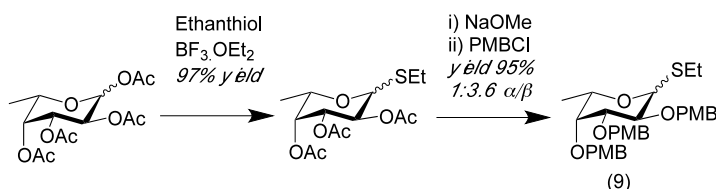
The LeX trisaccharide was synthesized starting from glucosamine (Scheme 10). The starting monosaccharide was functionalised with a cyanomethylthiol group and selectively protected at the 6-position (total yield over 5 steps 18.3%). A trichlorolactonitrile (TCA)-galactose donor was then synthesised from peracetylated galactose (Scheme 11) (77% yield over 2 steps). Finally *para*-methoxybenzyl (PMB) protected ethanethiol fucose donor was synthesised, yielding an α/β mixture (Scheme 12) (95% over two steps).



Scheme 10 - Synthesis of the glucosamine-based IME monosaccharide building block for Lewis-X



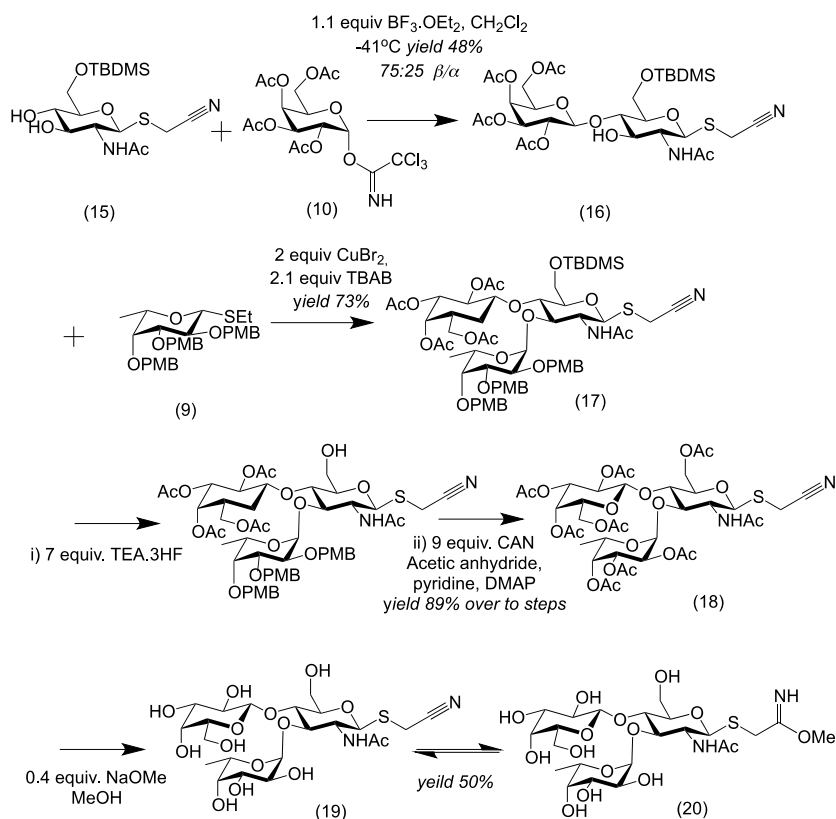
Scheme 11 - Synthesis of the galactose-based monosaccharide building block for Lewis-X



Scheme 12 - Synthesis of the fucose-based monosaccharide building block for Lewis-X

The first glycosylation reaction was conducted between the glucosamine cyanomethyl thiol functionalised monosaccharide and the

galactose TCA donor. The donor was regiospecific for the 4-position; despite the 3- and 4-hydroxy groups being unprotected (48% total yield, 36% β anomer). Surprisingly, an α/β mixture was produced, even though the neighboring group participation from the 2-acetyl group on the galactose should direct solely β -addition. NMR experiments revealed that this acetyl group was removed in the α -product. The second glycosylation was conducted between the remaining hydroxy at the 3-position on the β disaccharide and the fucose ethanethiol donor (either the α or β monosaccharide can be used as the donor) (73% yield). Finally, the PMB protecting groups were removed by oxidation with ceric ammonium nitrate (CAN), and the LeX trisaccharide was peracetylated (89% over two steps) and stored under an inter atmosphere at -20°C (23% total yield over all steps)(**Scheme 13**). The LeX was activated to IME-LeX under the same conditions as the previous monosaccharides.



Scheme 13 – The synthesis of the trisaccharide Lewis-X, functionalized at the anomeric position with a cyanomethyl thiol group. The final step in the synthesis

allows simultaneous deacylation and activation to the amine-reactive imidate form (IME-LeX).

To form the amine-reactive 2-imino-2methoxyethyl-1-thioglycosides (IME), the cyanomethylthio-sugars were dissolved in MeOH in the presence of catalytic amounts of NaOMe (**Scheme 13**). Schaefer and Peters first suggested in 1961 that imidate formation is an exothermic process⁷². This is consistent with the observations made by Stowell *et al* in the 1970s, who studied IME formation in a number of monosaccharides⁷³. They found that yields were lower at temperatures greater than 37°C and unchanged at temperatures lower than 20°C. The IME activation was therefore conducted at room temperature.

In order to study the kinetics of the equilibrium formed between cyanomethyl thiol LeX (**19**) and the IME-LeX (**20**), reactions were monitored by ¹H NMR. Deacetylation was rapid, and the reversible cyanomethylthiol-imidate reaction reached equilibrium after around 16 hours at room temperature (**Figure 3**). After 24 hours of reaction, it was noted that the percentage of IME began to decrease.

Initially, samples were either first neutralised with acid or dowex resin before the MeOH was evaporated or, once dry, dissolved in aqueous solvent and neutralised before addition to proteins. However, these methods gave limited levels of protein modification or showed no reactivity. Upon analysis by NMR, a new doublet peak was noted in the region of the glucosamine anomeric proton H-1 (**Figure 4**). The appearance of this new peak did not coincide with the observation of a new mass in mass spectrometric analysis (MS). Therefore, it was proposed that the LeX imidate underwent irreversible hydrolysis under neutral conditions. When the reaction was neutralized under anhydrous conditions, we saw complete return to the cyanomethylthiol and no hydrolysis product.

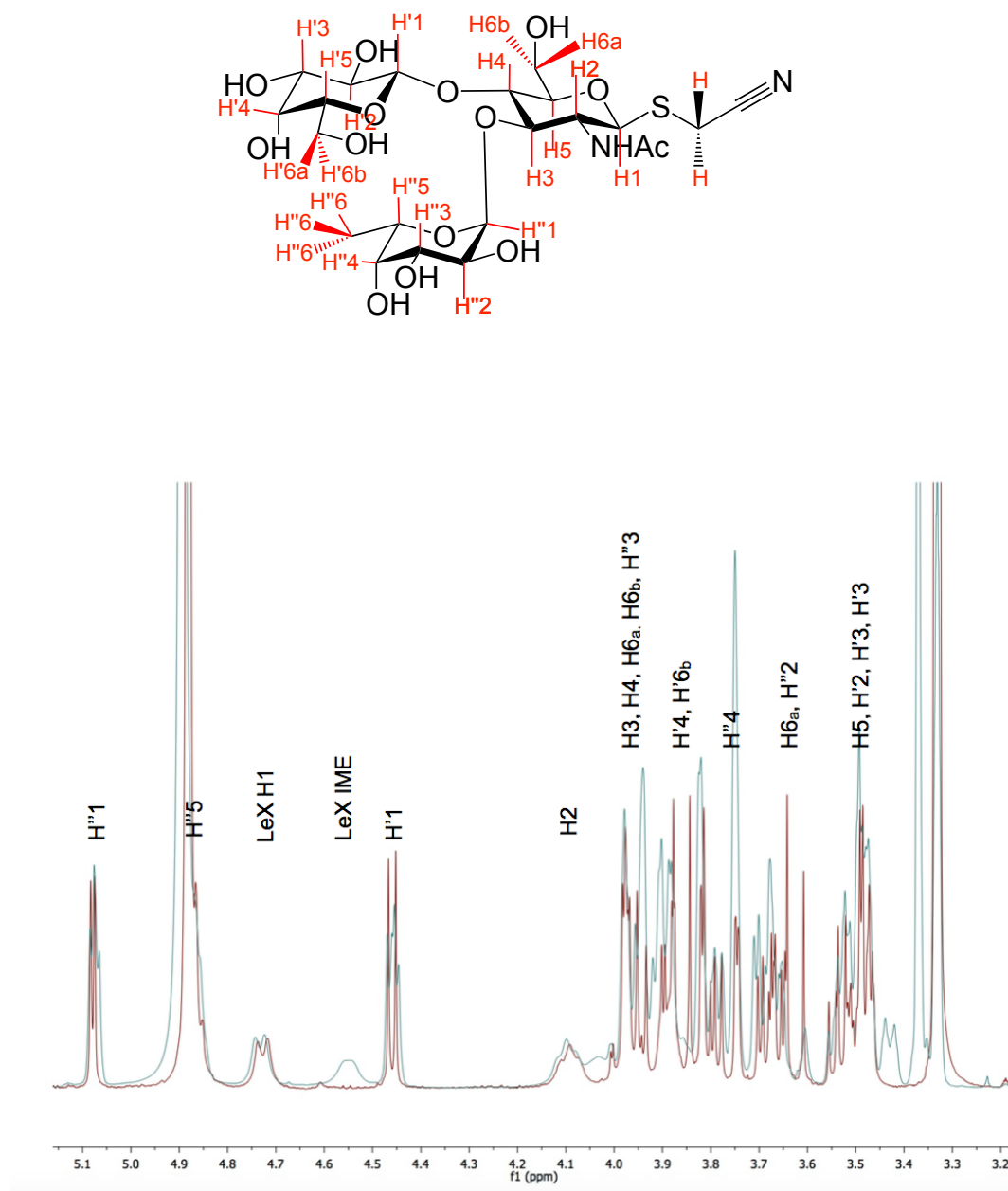


Figure 2 - An example of the proton NMR assignment of the equilibrium between cyanomethyl thiol LeX (Red) and 2-imino-2methoxyethyl-1-thiol (IME) LeX (Green). The proton nomenclature is also shown

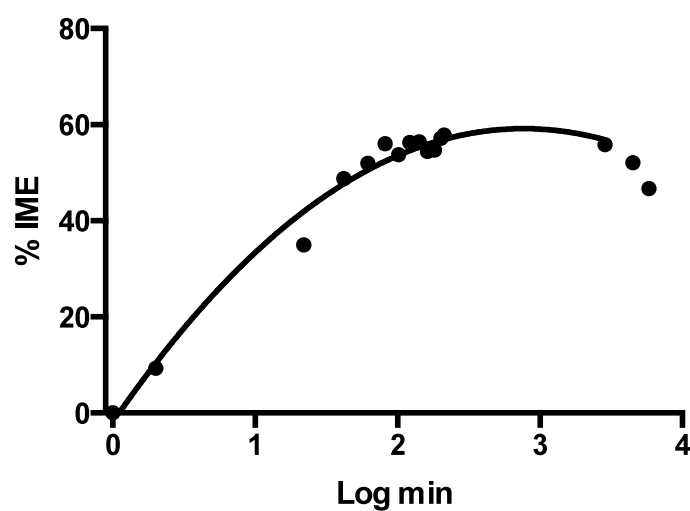


Figure 3 - The relative percentage of IME-LeX conversion over a time course after the addition of 0.4 equiv NaOMe in MeOD. The percentage of IME was ascertained using the deconvolute function of TopSpin. This allowed the measurement of the areas of the peaks of the IME-LeX H-1 relative to that of the LeX cyanomethyl thiol H-1.

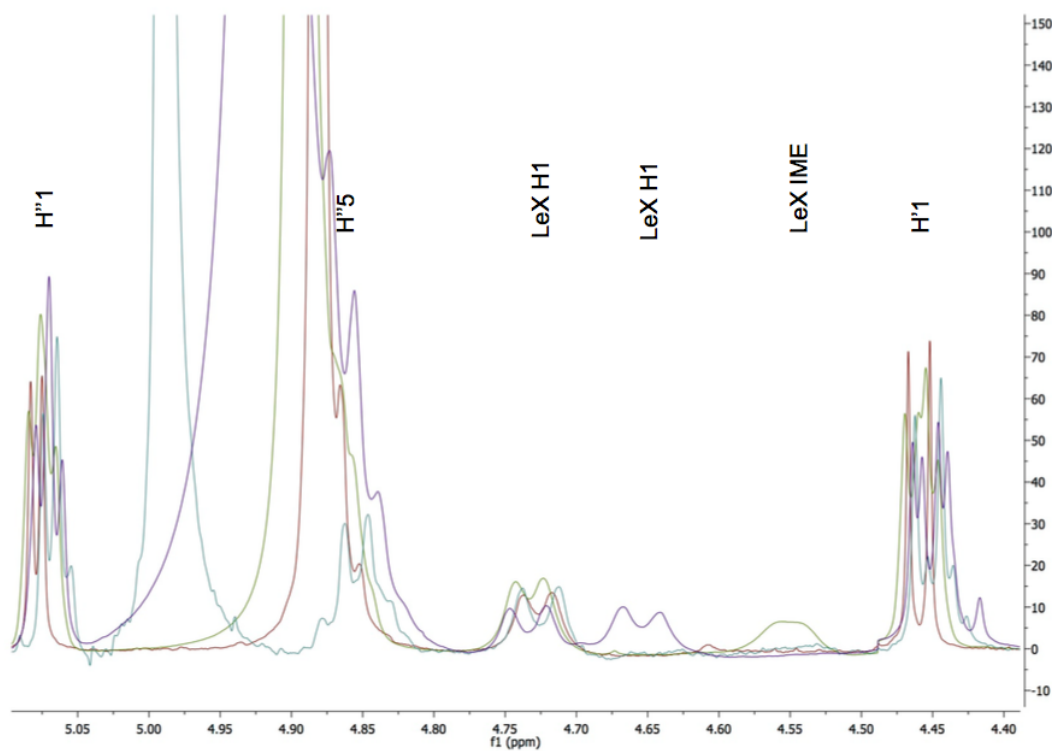


Figure 4 - An example of the proton NMR assignment of the equilibrium between cyanomethyl thiol LeX (Red) and 2-imino-2methoxyethyl-1-thiol (IME) LeX (Green) and the hydrolyzed LeX (purple).

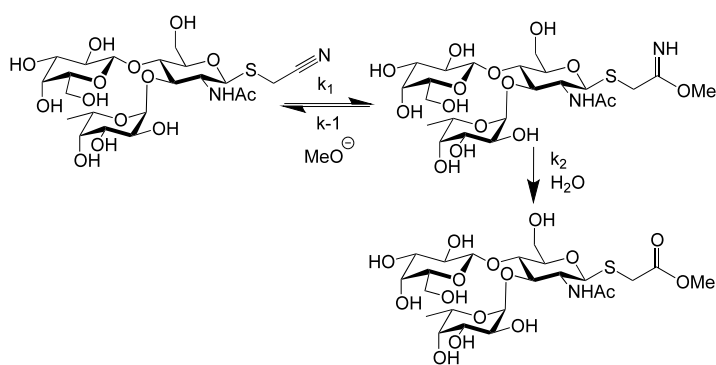
Makoff *et al* measured the rate hydrolysis of methyl acetimidate and found that it is a pseudo-first order reaction with respect to the methyl acetimidate⁷⁴. Further, this reaction is highly temperature- and pH-dependent. The rate of hydrolysis decreased with increased pH (**Figure 5**), leading the authors to conclude that the methyl acetamidine is attacked by an H₂O molecule rather than hydroxyl ion. Therefore, the extent to which the acetimidate is protonated is the most important factor in determining the rate of hydrolysis.

Temperature (°C)	pH	$10^{-2} \times k'$ (min ⁻¹) (corrected to pH 8.0)	Half-life ($t_{1/2}$) (min) (corrected to pH 8.0)	pH	$10^{-2} \times k'$ (min ⁻¹)	Half-life ($t_{1/2}$) (min)	Temperature (°C)	pH	k/k' (M ⁻¹)	k (min ⁻¹ ·M ⁻¹) (corrected to pH 8.0)
0	8.4	0.317 ± 0.113	220	6.8	2.42 ± 0.35	29	0	8.4	123 ± 22	0.293
10	8.2	0.768 ± 0.050	90	7.2	2.54 ± 0.12	27	10	8.2	124 ± 19	0.827
20	8.0	1.92 ± 0.22	36	7.6	2.30 ± 0.18	30	20	8.0	97.7 ± 10.0	1.88
30	7.8	3.74 ± 0.29	19	8.0	2.07 ± 0.17	33	30	7.8	88.9 ± 8.7	3.82
40	7.6	8.52 ± 0.41	8.1	8.4	1.59 ± 0.10	44	40	7.6	69.3 ± 8.6	7.82
				8.8	1.27 ± 0.05	55				

Figure 5- Data taken from Makoff *et al*⁷⁴, where k' is the rate of hydrolysis of methyl acetimidate and k is the rate of amidination of denature aldolase at 0.1m NaCl.

Under basic conditions, the presence of a small amount of water, such as contained within the 'wet' MeOH and NaOMe did not cause hydrolysis of the IME-LeX. Therefore we can assume, in accordance with Makoff *et al*, that the hydrolysis of the LeX imidate product was not performed by hydroxide.

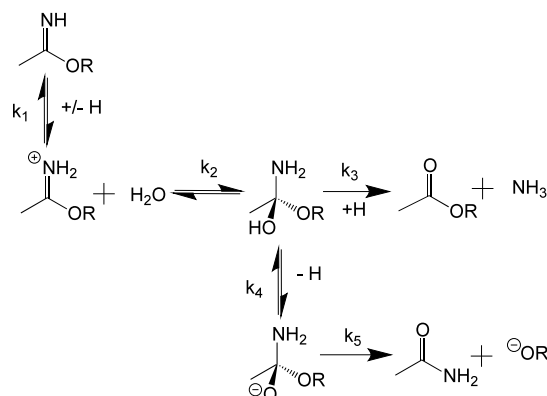
The following scheme (**Scheme 13**) was thus proposed:



Scheme 13- The hydrolysis of IME-LeX where $k_{-1} \gg k_1$ under neutral conditions.

However, when neutralized in aqueous conditions we observed hydrolysis of the imidate only. Thus, the concentration of the cyanomethyl thiol remains constant after neutralization, therefore $k_2 > k_{-1}$. Chaturvedi *et al*

propose the following mechanism for the hydrolysis of imidate esters (**Scheme 14**)⁷⁵.



Scheme 14 – A mechanism for the hydrolysis of imidate esters proposed by Chaturvedi et al⁷⁵.

The fact that the methyl ester, rather than the amide form of the LeX is observed suggests that in the case of the hydrolysis of the IME-LeX $k_3 > k_5$.

As the hydrolysed LeX did not react with proteins, steps were taken to avoid forming this product both during the imidate formation and the subsequent protein reaction. IME activation reactions were not neutralised before MeOH evaporation. However this posed another issue, IME suagrs samples caused a large increase in pH when dissolved in protein samples, under standard buffer conditions. It was thus necessary to conduct studies to define the pH optimum of the imidate-lysine reaction.

2.2.2 Imidate-lysine amidination reaction optimization.

It is probable that the IME-LeX is more stable to hydrolysis at $>\text{pH } 8$, as the pK_a of the acetimidate is around 7.5 ⁷⁴. The pK_a for the primary amino group on lysine is 10.5 , therefore the optimum pH for reaction is likely to be between these values. The reaction between IME-LeX and a model protein, hen egg lysozyme (HEL, $\text{MW}=14,304\text{Da}$), was monitored over a range of pH values. The extent of glycosylation was determined by mass spectrometric (MS) analysis and SDS-PAGE mass shift. The pH optimum for reaction was found to be 9 (**Figure 7**).

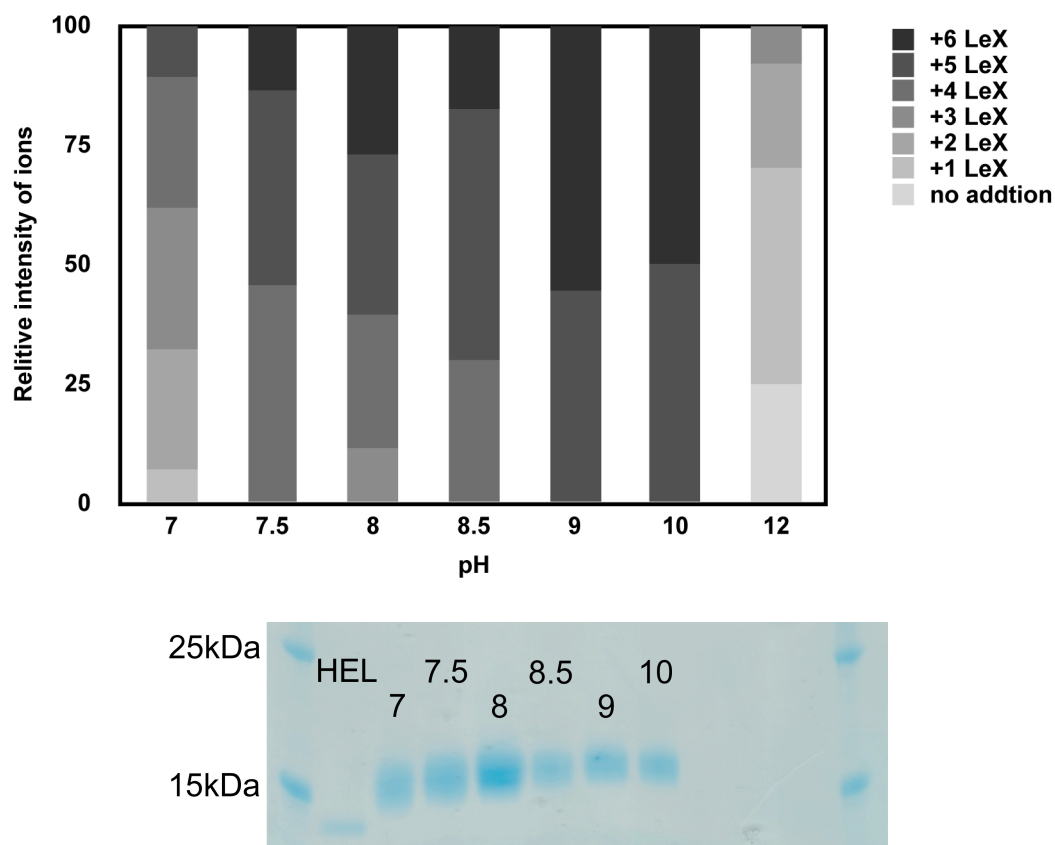
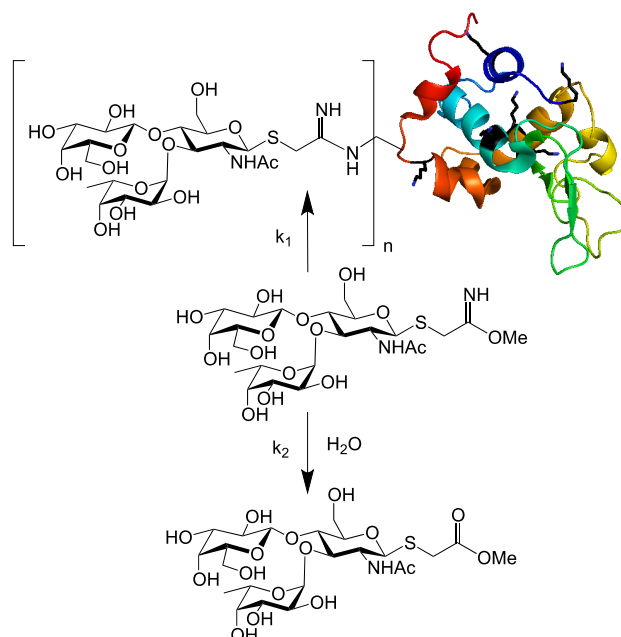


Figure 7 - HEL protein was modified with IME-LeX at different pH values. After 2 hours samples were taken and analysed by mass spectrometry and SDS-PAGE. The relative intensity of the deconvoluted protein ions was measured and plotted. See Figure E1 for examples of mass spectra. The greatest LeX conjugation to HEL was observed at pH 9.

The rate of amidination is likely the result of two opposing factors (**Scheme 20**). The desirable reaction involves a nucleophilic attack by an unprotonated lysine ϵ -amino group on the protonated acetimidate. However, exposure of the IME- LeX to the required aqueous environment at neutral pH causes irreversible hydrolysis. Makoff *et al* predicted the discovery that the optimum pH for protein-IME reaction is 9. Wolsy *et al* also conducted similar reactions at pH 8.5-9^{69,74}.



Scheme 20 - The irreversible reaction with 2-imino-2-methoxyethyl-1-thiol (IME) LeX with either the primary amine R groups of lysine residues or water.

The choice of buffer must also preclude any other chemical groups that may compete with the IME-LeX -lysine reaction. The glycosylation of HEL was conducted in either 0.1M PBS, borate and ammonium carbonate buffers at pH 8.5 and the reaction monitored by MS and SDS-PAGE mass shift (**Figure 8**).

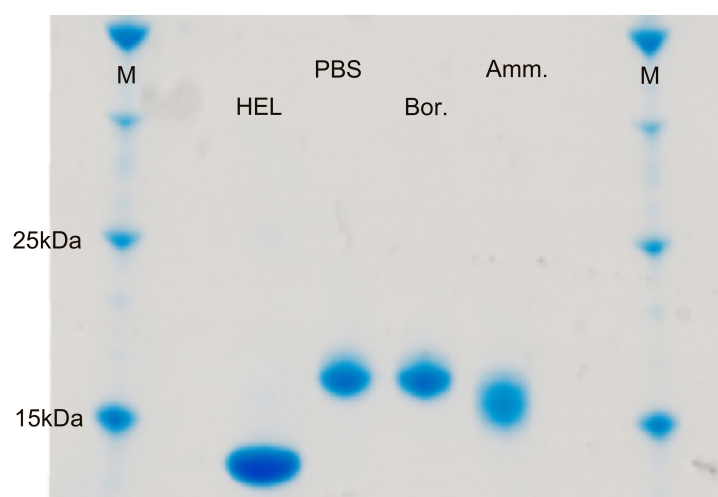


Figure 8 - SDS-PAGE of HEL protein modified with IME-LeX in PBS, borate or ammonium carbonate buffers. IME modification is less efficient in ammonium carbonate buffer.

Masses obtained for the reaction in sodium borate and PBS demonstrated similar results, namely, a major peak at 17813 Da (HEL + 6 LeX) and a minor peak at 18397 Da (HEL + 7 LeX). The major mass corresponds to all of the protein's lysine residues successfully modified with the LeX reagent. The minor peak bears an additional mass from the glycosylation of the N terminus of the protein. The reaction in ammonium carbonate shows masses at 16059 Da (HEL + 3 LeX), 16645 Da (HEL + 4 LeX), 17229 (HEL + 5 LeX) and 17814 (HEL + 6 LeX). The N terminus appears not to have been glycosylated in this buffer and the reaction overall less efficient, most likely due to ammonium species' possible competitive reaction with the LeX imidate. The ideal buffer was found to be N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) buffer as it has a pKa of 8.44 and does not contain any primary amine groups.

The reaction between IME-Man and HEL was conducted at pH 9 in TAPS buffer, at 75 and 35 equivalents of IME per lysine (6 on HEL). The reaction was monitored by SDS-PAGE and MS. The HEL mass was shown to have increased at 30 minutes, in a manner proportional to the equivalents of IME-Man employed (**scheme 9**). No further mass increases were observed at later time-points. The maximum glycosylation levels obtained at 35 equiv IME were 15,484Da (HEL+5 Man), 15,719Da (HEL+6 Man) and 15955Da (HEL+7 Man). At 75 equivalents all lysines had reacted, giving 15,719Da (HEL+6 Man), the major peak at 15955Da (HEL+7 Man) indicated significant *N*-terminus glycosylation. These promising results on a protein model system prompted us to investigate the glycosylation of Ad, described in the next chapter.

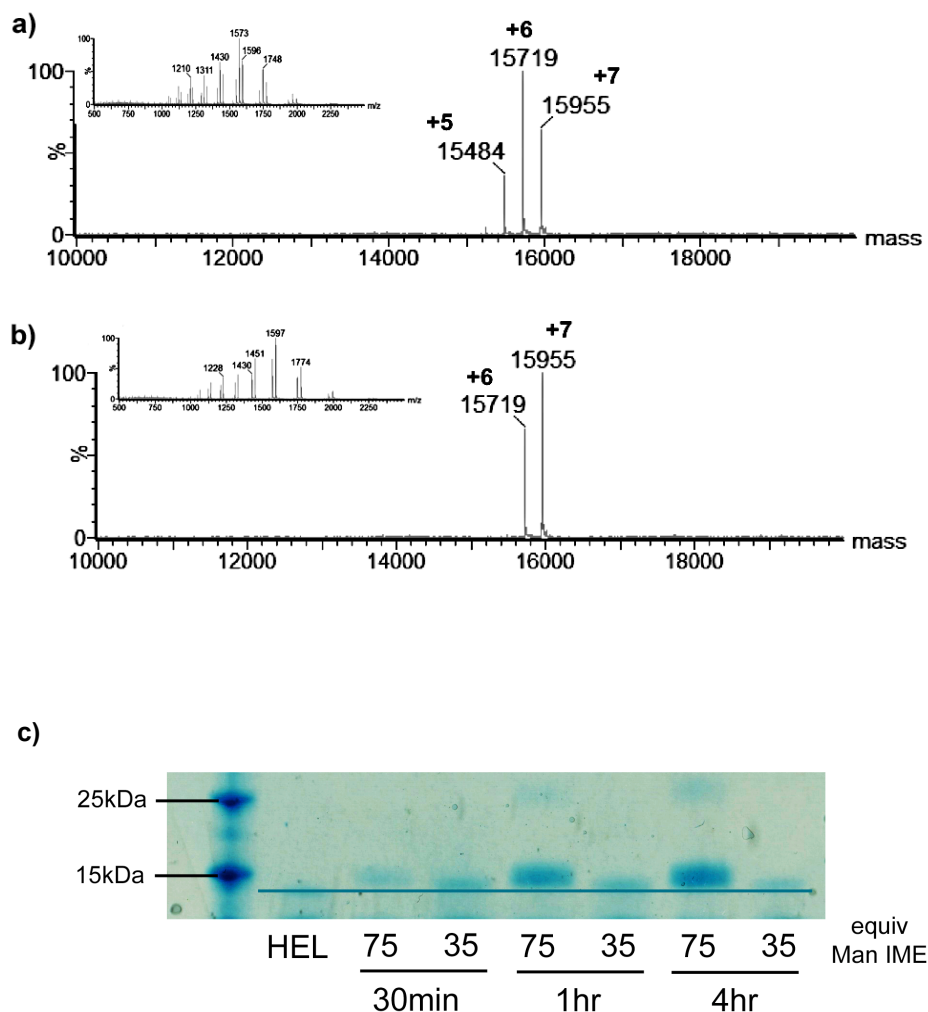


Figure 8 – Mass spectra and SDS-PAGE of HEL protein modified with IME-Man at 75 and 35 equivalents of IME/lysine (6 on HEL plus the *N* terminus). a) Reaction after 4 hours with 35 equivalents IME produced 5, 6 and 7 glycosylations. b) Reaction after 4 hours with 75 equivalents IME produced 6 and 7 glycosylations c). The reaction was monitored over 4 hours at room temperature. The mass shift was greater at 75 then 35 equivalents and the reaction finished within the first 30 minutes. The blue bar represents the mass shift level of unmodified HEL

2.3 Discussion

Cyanomethylthiol functionalised mannose, galactose and LeX were successfully synthesised. By reaction with NaOMe the cyanomethylthiol group was converted to the amine-reactive IME. Equilibrium was established between the cyanomethylthiol and IME affording 50% conversion after 16 hours. In theory, the cyanomethylthiol and the IME

could be separated and the thiol recovered, although this was not attempted. The IME product was found to be susceptible to hydrolysis at neutral pH and therefore was not neutralised before drying. Using 0.6M of TAPS buffer was shown to solve the issue of increased basicity upon IME addition to protein samples. The pH optimum for the lysine-IME reaction was found to be between 8.5 and 9. Man, Gal and LeX IME sugars were successfully conjugated to the model HEL protein, achieving up to 100% lysine coverage, with *N*-terminal reaction also observed. No additional glycosylations were detected. Further studies could expand on the observed details of lysine-IME reactivity, perhaps in concert with mass spectrometry mapping of glycosylation site to identify privileged sites of reaction.

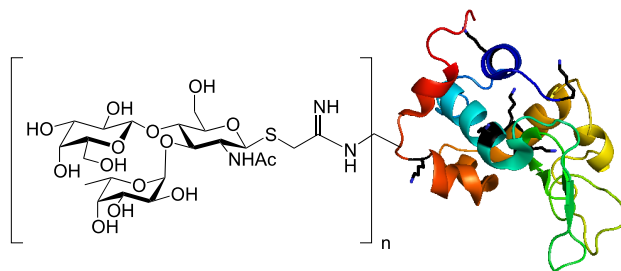
2.4 Experimental

General considerations. Low resolution mass spectra (LRMS) were recorded on a Waters Micromass LCT Premier TOF spectrometer using electrospray ionization (ESI), m/z values are reported in Daltons. Proton nuclear magnetic spectra (^1H NMR) and carbon nuclear spectra (^{13}C NMR) were recorded on a Bruker DQX 400MHz spectrometer. NMR shifts were assigned using COSY and HSQC spectra. All shifts are quoted on the δ scale in ppm using residual solvent as the internal standard. Coupling constants (J) are reported in Hz with the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter with a path length $l=10\text{cm}$. The rotations are quoted with implied units of $10^{-1} \text{ deg cm}^2 \text{ g}^{-1}$. Concentrations (c) are given in $\text{g}/100\text{mL}$. Thin layer chromatography (TLC) was carried out on aluminum sheets coated with 60F254 silica gel (Merck). TLC visualization was achieved via a UV lamp and/or ammonium molybdate (5% in 2M H_2SO_4). Flash chromatography was carried out on Geduran SI 60 ($40\text{-}63\mu\text{m}$) silica (Merck). PE solvent is light petroleum ether boiling in the range $40\text{-}60^\circ\text{C}$. Anhydrous solvents were purchased from Sigma Aldrich and were stored over molecular sieves. Other solvents were analytical or HPLC grade and were purchased from Sigma Aldrich and used without prior purification. Brine refers to a saturated NaCl solution.

IME-LeX reaction with HEL – pH variation . A buffer solution of 0.1M of each MOPs, HEPES and Sodium borate was made and then adjusted to pH values 7, 7.5, 8, 8.5, 9, 9.5 and 10 using NaOH. 3.3mg of IME-LeX (6.6g of the mixture crude **19/20**) (75 equiv IME/ lysine (**6**)). Crude 1:1 mixture was neutralize with dowex resin before being quickly dried under high vacuum. This solid was then dissolved in $7.5\mu\text{L}$ of pH specific buffer. This was repeated for all buffer pH values any change in the pH was checked for using pH paper. To these solutions $5\mu\text{L}$ of HEL stock solution ($5\text{mg}/\text{mL}$ in buffer at pH) and the reaction was left for 1 hour at room temperature with

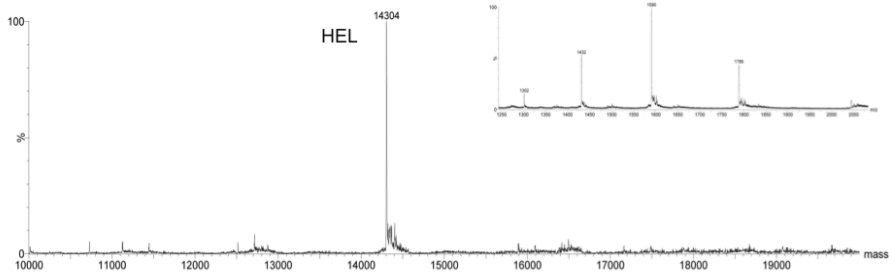
shaking. The modified HEL was then separated from the reaction solution using Vivaspin (3kDa filter, 13.2 rpm, 4°C, 20 minutes), and were all exchanged into the PBS solution, concentrated to the final volume of 100µL. The reaction was assessed using SDS PAGE (12% MES by Novagen) stained with Coosamine stain (Sigma Aldrich) and MS.

IME-LeX reaction with HEL

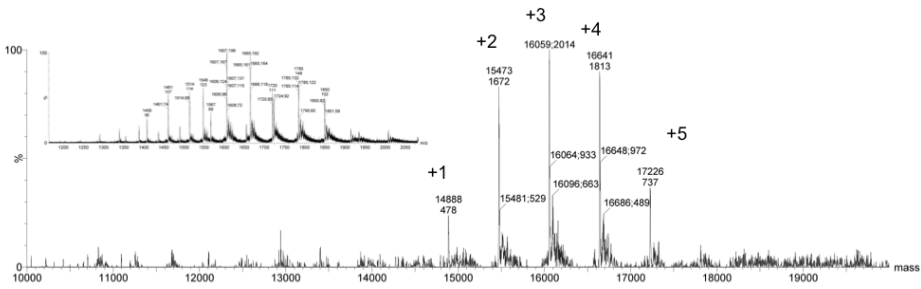


5.00mg of HEL was dissolved in 1mL (5mg/mL) of the PBS (50mM pH 8). 3.49mg of IME-LeX (7mg of 1:1 mixture **19/20**) (75 equiv/lysine) was dissolved in PBS (5mg/mL). To which was added 5µL of the HEL stock solution (5mg/mL) was added and the reaction was left at room temperature for 4 hours, monitored by SDS-PAGE. 1µL reaction mixture, 9µL MilliQ water 2µL loading buffer, was loaded into the wells and the gel (12-14% BrisTris NovexMinicell) us run with Perfect Protein marker (15-150kDa by Novagen®), MOPS running buffer at 200v of 55 minutes. The protein was visualized with Coomassie Blue stain. The modified HEL was then separated from the reaction solution using Vivaspin (3kDa filter, 13.2 rpm, 4°C, 20 minutes), and were all exchanged into the PBS solution, concentrated to the final volume of 100µL.

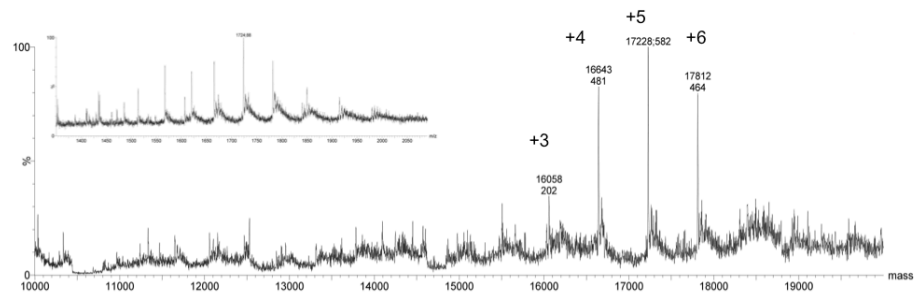
a)



b)



c)



d)

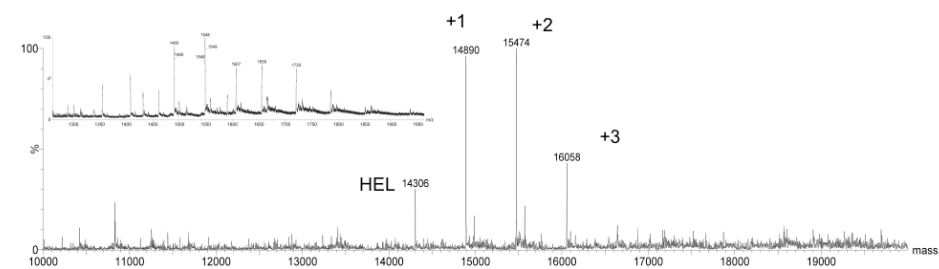
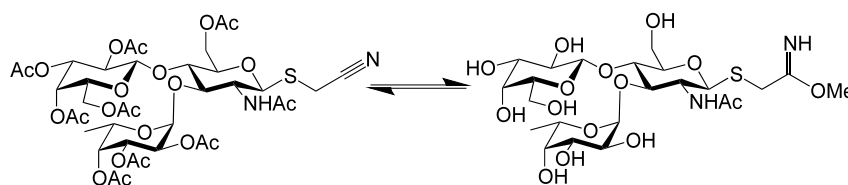


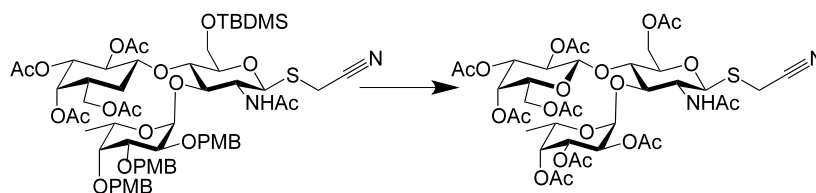
Figure E1 - Example of mass spectra from IME-LeX modified HEL protein samples, both deconvoluted mass and relative ion intensities are shown. The ion series are also shown as smaller plots. a) HEL protein, b) reaction with IME-LeX at pH 7, c) reaction with IME-LeX at pH 8, d) reaction with IME-LeX at pH 12.

α -L-Fucopyranosyl-(1-3)-[β -D-galactopyranosyl-(1-4)]-2-N-acetamido-2-deoxy-1-thio-(S-cyanomethyl)- β -D-glucopyranoside (19) and a -L-fucopyranosyl-(1-3)-[β -D-galactopyranosyl-(1-4)]-2-N-acetamido-2-deoxy-1-thio-(S-2-imino-2-methoxyethyl)- β -D-glucopyranoside(20)



51mg of the **18** was suspended MeOH (10mL/mM) to which was added 57 μ L NaOMe (25% in MeOH) (0.025mM 0.4 equiv of NaOMe). The reaction left stirring at RT for 16 hours, monitored for product formation by mass analysis. The reaction was stopped by solvent evaporation, 30 minutes N₂ flow following by 2 hours under high vacuum. **R_f** (Actone:water 8:2)= 0.85 cyanomethyl thiol and =0.57 imidate **LR-ESI-TOF-MS m/z**: [(**19**)+Na]⁺=607.1 gmol⁻¹, [(**20**)+H]⁺=617 gmol⁻¹ [(**20**) Na]⁺=639.2 gmol⁻¹ **HR-ESI-TOF-MS m/z**: [(**19**)+Na]⁺ =607.1779 (calculated=607.1779) gmol⁻¹, gmol⁻¹ [(**20**) Na]⁺=639.2045 gmol⁻¹ (calculated=639.2042). **¹H NMR** (500MHz CDCl₃) δ (ppm): 5.08 (d, J=4.00Hz, 1H, H''-1), 4.86 (m, 1H, H''-5), 4.46 (d, J= 10.16Hz, 1H, H-1 LeX - IME **20**), 4.75 (d, J=10.04Hz, 1H, H-1 cyanomethyl thiol LeX **19**), 4.10 (m, 1H, H-2), 3.98 (m, 3H, H''-3, H-3, H-4), 3.93 (d, J=9.45Hz, 1H, H-6), 3.90 (m, H-2, H-5, H'-5), 3.84 (m, 1H, SCH₂CH), 3.79 (m, 1H, H'-6a), 3.74 (m, 1H, H'-4), 3.70 (m, 3H, H''-4, H-2, H'-6b), 3.64 (d, J=17.02Hz, 1H, SCH₂CH), 3.55 (m, 4H, H''-2, H'-2, H-5, H'-3), 1.98 (s, 3H, OAc), 1.21 (d, J=6.60Hz, 3H, CH₃). **¹³C NMR** (500MHz CDCl₃): 173.72 (OAc), 118.55 (CN), 103.96 (C-1'), 100.57 (C-1''), 84.64 (C-1), 81.95 (C-5), 76.75 (C-3), 74.96 (C-5'), 73.0 (C-4), 72.75 (C-3'), 71.16 (C-3''), 69.75 (C-5''), 62.86 (C-6'), 61.40 (C-6), 55.88 (C-2), 22.91 (OAc), 16.63 (C-6''). **M.P** 203-207°C.

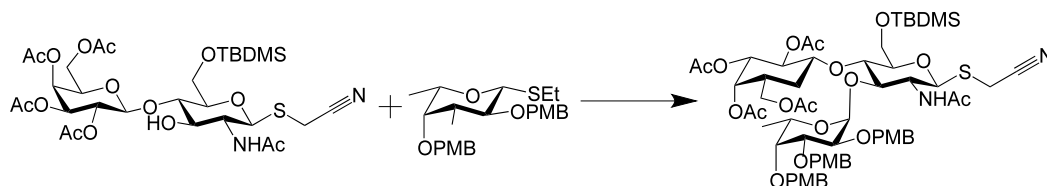
2,3,4-Tri-O-acetyl- α -L-fucopyranosyl-(1-3)-[2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl-(1-4)]-2-N-acetamido-6-O-acetyl-2-deoxy-1-thio-(S-cyanomethyl)- β -D-glucopyranoside (18)



17 (5.26) was dissolved in anhydrous THF (65mL) and triethylamine trihydrofluoride (4.90mL, 29.96mM, 7 equiv) after stirring over night at room temperature the solvents were evaporated. The crude mixture was purified by flash chromatography on silica (EtOAc:PE 7:3, 8:2, 9:1) to afford the TBDMS deprotected sugar as a white solid (4.60g 95% yield), R_f (EtOAc:PE 9:1)= 0.26 **LR ESI-TOF-MS** m/z : $[M-Na]^+ = 1136.41$. This solid (4.60g 4.08mM) was dissolved in acetonitrile (75mL, 18mL/mM) and water (8.3mL, 2mL/mM). CAN was then added (13.58g, 6 equiv) and the solution stirred at room temperature for 2 hours, the additional CAN (6.79g, 3 equiv, total of 9 equiv. 37.10mM) was added and the reaction left for 3 hours. The reaction was then cooled to 0°C before acetic anhydride (300mL), pyridine (300mL) and DMAP (50mg, 0.41mM, 0.10 equiv) were added slowly. The reaction was allowed to stir overnight at room temperature before MeOH (100mL) was added slowly. The solvents were then evaporated and the crude product co evaporated with toluene three times before purification by flash chromatography on silica (EtOAc:PE 1:1, then 7:3 finally 8:2) to afford **18** (2.01g, 50.4% yield over two steps) as a white amorphous solid. R_f (EtOAc)= 0.60 **HR-ESI-TOF-MS** m/z : $[M-Na]^+ = 943.2626$ (calculated= 923.2625) **LR-ESI-TOF-MS** m/z : $[M-Na]^+ = 943.25$, $[M-H]^- = 919.23$, $[M+Cl]^- = 955.31$, $[\alpha]_D^{25} -102.6$ (c 0.5, $CHCl_3$), 1H NMR (400MHz $CDCl_3$) δ (ppm): 6.15 (d, $J=9.48$ Hz, 1H, NH), 5.39 (br 3H, $H''-1$, $H''-4$), 5.18 (dd, $J=10.90$ Hz, $J=2.62$, $H1$, $H''-3$), 5.03-4.92 (m, 3H, $H'-2$, $H'-3$, $H''-5$, $H''-2$), 4.96 (br, 2Hm $H-6a$, $H-1$), 4.66 (m, $H1$, $H'-6a$), 4.50 (br, s, $H-1$, $H'-1$), 4.31 (dd, $J=10.08$ Hz, $J=7.92$ Hz, $H1$, $H'-6b$), 4.19 (br $H1$, $H-2$), 4.04 (m, 1H, $H-6b$), 3.90 (m, 3H, $H'-5$, $H-5$, $H-4$), 3.63 (b, $J=17.05$ Hz, 1H, SCH_2CH), 3.55 (br, 1H, $H-3$), 3.29 (d, $J=17.05$ Hz, 1H, SCH_2CH), 2.17-1.80 (all, s, 27H, 9xOAc), 1.23 (d, $J=6.16$ Hz, 1H, $H''-6$). ^{13}C NMR (400MHz $CDCl_3$): 116.39 (CN), 100.46 (C-1'), 95.63 (C-1''), 82.64 (C-1), 77.18 (C-3), 74.38 (C-5), 71.29 (C-4), 71.02 (C-5''), 68.91 (C-2'), 67.82 (C-

3'), 67.80 (C-3''), 66.65 (C-4''), 64.27 (C-2''), 61.39 (C-6'), 23.25-20.53 (9xOAc), 14.29 (SCH₂CN), 15.82 (C-6''). **M.P** 148.1-150.2°C

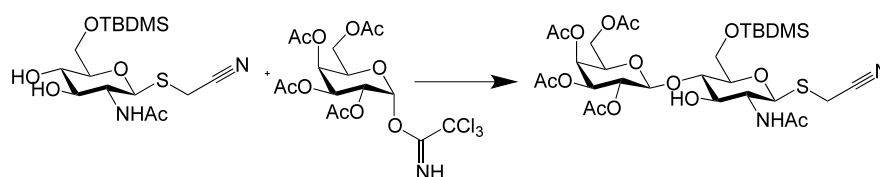
2,3,4-Tri-*O*-*para*-methoxybenzyl- α -L-fucopyranosyl-(1-3) -[2,3,4,6-tetra-*O*-acetyl- β -D- galactopyranosyl-1-4)] -2-*N*-acetamido-6-*O*-*tert*-butyldimethylsilyl-2-deoxy-1-thio-(*S*- cyanomethyl)- β -D-glucopyranoside (17)



8.63g (15.18mM, 2 equiv) of donor **9** and 5.47g (7.58mM) of the acceptor **16** were co-evaporated with toluene (three times 100mL), dried under vacuum and dissolved in 1:1 DCM: DMF (30mL/mM acceptor) to which freshly activated powdered 4 Å MS were added (4mg/mg acceptor) and was left stirring under argon at room temperature for 1 hour. Next 3.39g (15.18 mM, 2 equivalents) CuBr₂ and 5.14g (15.93 mM, 2.1 equivalents) Bu₄NBr were added and stirred for 20 hours, protected from light at room temperature. The reaction was then filtered over celite and rinsed with DCM (300mL), the filtrate was then washed with aqueous NH₄Cl (seven times 100mL, pH 8.5) and once with brine (200mL). The aqueous phase was back extracted with DCM (3x100mL) and the combined organic phases were washed with brine and dried over MgSO₄, filtered and concentrated in vacuo to yield a yellow oil. The product was then purified by flash chromatography (Petrol:EtOAc, 6:4, 5:5, 4:6 and 3:7) yielding white foam (5.34g 56% yield from **16**). **R_f** (EtOAc) acceptor= 0.8, Donor = 0.45, product = 0.67, [α]_D²⁵ -35.8 (c 0.5, CHCl₃), **LR-ESI-TOF-MS m/z**: [M-Na]⁺=1249, [M-H]⁻=1225, **¹H NMR** (400MHz CDCl₃) δ (ppm): 7.26-7.35 (6H, m, PMB-H) 6.80 - 6.93 (6H, m, PMB-H), 6.17 (1H, d, J=7.00Hz, NH), 5.38 (1H, br d, J=3.16Hz H-4), 5.12 (2H, m, H''-1, H'-2), 4.96 (dd, 1H, H'-3 J=10.52, J=3.52), 4.91 (m, 2H, CH₂bz), 4.85 (m, 1H, H-1), 4.82 (m, 1H, H-2), 4.77 (d, 1H, H-1'), 4.71 (m, 2H, CH₂bz), 4.69 (d 2H, J=11.10Hz CH₂bz), 4.63 (d, 2H, d,

$J=11.00\text{Hz}$, CH_2bz), 4.31 (m, 1H, $\text{H}''\text{-5}$) 4.13-3.99 (m, 5H, H-6a, $\text{H}''\text{-3}$, $\text{H}''\text{-2}$, H-6b, H-3), 3.90 (m, 4H, H-6a and b, H-5, $\text{H}''\text{-4}$), 3.81-3.80 (m, 9H, 3xMe), 3.66 (br. s, 1H, $\text{H}'\text{-4}$), 3.48 (d $J=17.17\text{Hz}$, 1H, SCH_2CN), 3.34 (m, 1H, $\text{H}'\text{-5}$), 3.16 (d, $J=17.15\text{Hz}$, 1H, SCH_2CN), 2.06, 2.02, 1.98 (3xs, 12H, 3xOAc), 1.75 (s, 3H NHOAc), 1.17 (d, $J=6.56\text{Hz}$, 3H, $\text{H}''\text{-6}$), 0.91 (s, 9H $(\text{OCH}_3)_3$), 0.11 (m, 6H $(\text{CH}_3)_2$). ^{13}C NMR (400MHz CDCl_3) δ (ppm): 130-128 (PMB), 116-113 (PMB), 99.3 ($\text{C}'1$), 98.4 ($\text{C}''1$), 81.7 ($\text{C}1$), 81.5 ($\text{C}3$), 80.2 ($\text{C}'5$), 79.7 ($\text{C}5$), 76.7 ($\text{C}'4$), 76.4 ($\text{C}''3$), 76.2 ($\text{C}'3$), 74.0, 73.8, 73.0 (CH_2Bz), 73.0 ($\text{C}''2$) 70.6 ($\text{C}''4$), 70.5 ($\text{C}'3$), 68.9 ($\text{C}'2$), 67.0 ($\text{C}4$), 66.8 ($\text{C}'5$), 61.5 ($\text{C}'6$), 60.6 ($\text{C}6$), 55.3-55.24 ($\text{SC}(\underline{\text{CH}}_3)$), 25.8 (OMe), 23.1 (NHOAc), 28.4, 20.6, 20.5 (OAc), 16.8 ($\text{C}''6$), 14.3 (SCH_2CN), -5.0 (SMe), -5.23 (SMe).

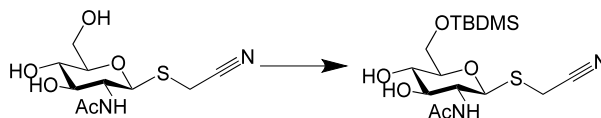
2,3,4,6-Tetra-*O*-acetyl- α -D-galactopyranosyl-(1-4)-2-*N*-acetamido-6-*O*-*tert*-butyldimethylsilyl-2-deoxy-1-thio-(*S*-cyanomethyl)- β -D-glucopyranoside (16)



10.10g (25.88 mM) of acceptor **15** and 13.98g (28.4 mM, 1.1 equiv.) of donor **10** were co evaporated with toluene (three times 100mL), dried under vacuum and then dissolved in anhydrous CH_2Cl_2 (10mL/mM acceptor) to which freshly activated powdered 4 Å MS were added (2.5mg/mg acceptor). The solution was stirred for 1.5 hours at room temperature under argon followed by cooling to -41°C using a dry ice acetonitrile bath. BF_3OET (3.5mL, 29.32 mM, 1.1 equivalent) was added slowly and stirred for 2 hours. 5mL of Et_3N was added to quench the reaction, which was filtered over celite washed three times with 100mL DMC. The filtrate was dried under vacuum. The crude mixture was then purified to flash chromatography on silica (using, petrol:EtOAc 3:7, 2:8, 1:9 and neat EtOAc). To yield a white foam (5.47g, 29% yield β anomer). R_f (EtOAc) Acceptor = 0.75, Donor = 0.1, product = 0.62, $[\alpha]_{\text{D}}^{25}$ -27.8 (c 0.5, CHCl_3), LR -ESI-TOF-MS m/z =

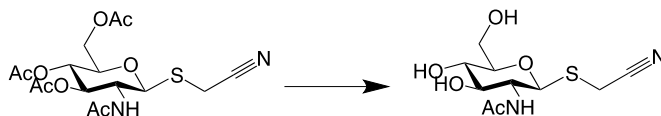
$[M+Na]^+ = 743.17$, $[M+H]^+ 721.28$, $[2M+Na]^+ = 1463.30$, 1H NMR (400MHz $CDCl_3$) δ (ppm): β anomer 5.51 (d, $J=8.68Hz$, 1H, NH), 5.41 (d, $J=2.88Hz$ 1H $H'-4$) 5.24 (dd, $J=10.38$ and 8.10 , 1H, $H'-2$), 4.98 (dd, $J=10.48$ and 3.36 , 1H, $H'-3$), 4.70 (d, 1H $J=10.28Hz$ H-1), 4.63 (d, $J=8.08Hz$ 1H, $H'-1$) 4.14-4.16 (m 2H $H'-6$) 4.00 (t, $J=6.80Hz$ 1H, $H'-5$), 3.84-3.91 (m, 2H H-2 and H-6a), 3.68-3.77 (m 3H H-3, H-4, H-6b), 3.58 (d, 1H $J=16.92Hz$ SCH_2CN), 3.39-3.42 (m 1H, H-5), 3.34 (d, $J=16.92Hz$ 1H, SCH_2CN), 2.17, 2.08, 2.07, 2.05, 2.04 and 2.00 (s, 15H, $COCH_3$), 0.93 (s, 9H, $Si(CH_3)_3$), 0.10 (2 x s, 2 x 3H, $SiCH_3$). ^{13}C NMR (400 MHz $CDCl_3$) 101.47 ($C'-1$), 82.64 (C-1), 80.31 (C-4), 78.88 (C-5), 73.38 (C-3), 71.35 ($C'-5$), 70.80 ($C'-3$), 68.56 ($C'-2$), 66.77 ($C'-4$), 61.33 ($C'-6$), 61.19 (C-6), 54.32 (C2), 25.83 (CH_3)₃, 23.31 (OAc) 20.70 (OAc) 14.34 (SCH_2CN) -4.97 (CH_3).

2-N-Acetamido-6-O-*tert*-butyldimethylsilyl-2-deoxy-1-thio-(S-cyanomethyl)- β -D-glucopyranoside (15)



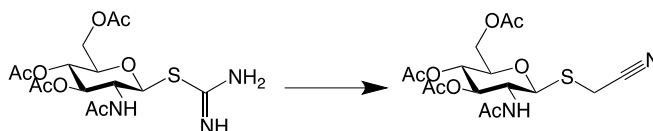
14 (4.00g, 14.48mM) was dried by toluene evaporation (3x50mL) and high vacuum. The crystals were then dissolved in 7mL of anhydrous DMF and cooled to $0^\circ C$. Imidazole (1.47g, 21.72mM, 1.5 equiv) and *tert*-Butyldimethylsilyl chloride (TBDMS-Cl) (2.40g, 15.93mM 1.1 equiv) were added to the solution. After 16 hours the solvents were evaporated and the product purified by flash chromatography (EtOAc neat, EtOAc:MeOH 9:1) To yield **15**, colorless crystals (1.96g, 4.93mM 34% yield) R_f (9:1 EtOAc:MeOH) = 0.44. $[\alpha]_D^{25} -59.8$ (c 0.5, MeOH) LR-ESI-TOF-MS m/z := 389.1 $[M-H]^-$ 425.0 $[M+Cl]^-$ 1H NMR (400MHz $CDCl_3$) δ (ppm): 7.36 (d $J=9.16Hz$, H1, NH), 4.71 (d $J=10.29Hz$, H1 H-1), 4.03 (m, H1, H6b) 3.89-3.82 (m, H2 H-2, H-6a), 3.76 (d $J=16.92Hz$, H1, SCH_2CN), 3.67 (d, $J=16.92Hz$, 1H, SCH_2CN) 3.64 (m, 1H, H-5), 3.50 (m, H1, H-4), 3.36-3.34 (m, H1, H-3) 1.81 (s, 9H (CH_3)₃) 0.051 (d, $J=4.12Hz$, 6H, (CH_3)₂) ^{13}C NMR (400 MHz $CDCl_3$): 83.60 (C-1), 82.54 (C-3), 76.52 (C-5), 71.02 (C-4), 63.29 (C-6), 54.81 (C-2), 22.67 (CH_3)₃, 14.03 (SCH_2CN), -5.44 (CH_3)₂. **M.P** $181^\circ C$

2-N-Acetamido-2-deoxy-1-thio-(S-cyanomethyl)- β -D-glucopyranoside (**14**)



12.58g (31.2 mM) of **13** was added to 250mL of anhydrous methanol to give a suspension, to which was added triethylamine (22.25mL, 159 mM, 5.6 equiv). The suspension was left to stir for 24hr at room temperature under argon and monitored by TLC. The solvents were evaporated and crystals dried in high vacuum overnight **14** (8.65g 100%) [α]_D²⁵ -65.0 (c 1, H₂O) **LR-ESI-TOF-MS** m/z : = 299.1 [M + Na]⁺, 275 [M - H]⁻ **¹H NMR** (400MHz CDCl₃) δ (ppm): 7.90 (d J=9.2 Hz, H1, NH), 5.13-5.09 (m 2H, OH-3, OH-4), 4.21-4.51 (m 2H, H-1, OH-6), 3.85 (d J=16.9Hz, 1H, SCH₂CN), 3.73 (d J=16.90Hz, H1, SCH₂CN), 3.69 (m, H1, H6b), 3.59 (q J=9.8Hz, 1H, H-2), 3.32 (m, 1H, H6a), 3.28 (m, 1H, H-3) 3.13 (m, 2H, H-4, H-5), 1.81 (s, 3H, AcO). **¹³C NMR** (400 MHz CDCl₃): 84.01 (C-1), 82.27 (C-5), 75.79 (C-3), 71.29 (C-4), 62.11 (C-2), 23.76 (OAc) 14.67 (SCH₂CN).

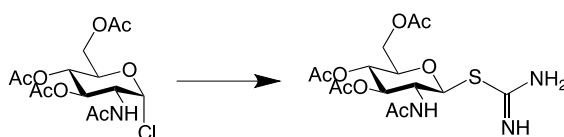
2-N-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thio-(S-cyanomethyl)- β -D-glucopyranoside (**13**)



20g of **12** (45 mM, 1 equiv) was dissolved in 1:1 water:acetone (240mL) to which was added sodium metasilphate (18.97g, 99.8mM, 2.2 equiv). potassium carbonate (8.08g, 58.5mM, 1.3 equiv) and chloroacetonitrile (54.12mL, 0.855m, 19 equiv). The solution was stirred under argon for 16 hours at RT. The completed reaction was then poured over 500mL of ice water forming white precipitate. The product was extracted by washing three times with DCM (250mL). The organic layer was then washed with ice-cold brine (2x250mL), filtered and the solvents evaporated to yield **13** a white amorphous solid (12.58g, 69%). [α]_D²⁵ -141.2 (c 0.5, CHCl₃) **R_f** (4:1 EtOAc : MeOH) = 0.72. **LR-ESI-TOF-MS** m/z : = 425.08 [M + Na]⁺, 827.12

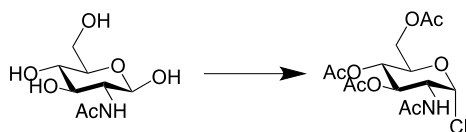
[2M + Na]⁺, 437.09 [M + K - 2H]⁻, 839.19 [2M + Cl]⁻ ¹H NMR (400MHz CDCl₃) δ (ppm): 6.01 (d, 1H, NH), 5.21 (m, 1H, H-3) 5.13 (m, 1H, H-4) 4.81 (d, J=10.37, 1H, H-1), 4.23 (m, 3H, H-2, H-6a and b), 3.81 (m, 1H, H-5), 3.70 - 3.31 (dd, 2H, SCH₂CN), 2.09, 2.05, 2.04, 1.97 (4 × s, 3H, OAc) ¹³C NMR (400 MHz CDCl₃): 116 (CN), 82.97 (C-1), 73.26 (C-3), 68.07 (C-4), 61.86 (C-6) 52.81 (C-2), 23.13, 20.74, 20.66 and 20.59 (4 × OAc) 14.65 (SCH₂CN)

2-N-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thiouronium-β-D-glucopyranoside hydrochloride (12)



20g of **11** (54.68mM, 1 equiv) was dissolved in anhydrous acetone prior to the addition of thiourea (8.32g, 109.36mM, 2equiv). This solution was then refluxed at 80°C under an argon atmosphere for 2 hours. After this time a white precipitate had formed, these crystals were separated by Buchner filtration and were washed with ice-cold ethanol. They were then dried under high vacuum overnight to yield **12** as white crystals (20g, 90% yield). **R_f** (100% MeOH = 0.34). [α]_D²⁵ -24.4 (c 0.5, H₂O) LR-ESI-TOF-MS **m/z**:- 330.10 [M + H]⁺, 811.16 [2M + H]⁺, 440.09 [M + Cl]⁻, 845.21 [2M + Cl]⁻ ¹H NMR (400MHz DMSO) δ (ppm): 9.49 (s, 2H, SCHNHNH₂), 9.30 (s, 2H, SCHNHNH₂) 8.46 (d, 1H, NH) 5.73 (d, J=10.48, 1H, H-1), 5.13 (m, 1H, H-3), 4.92 (m, 1H, H-4), 4.27 (m, 1H, H-5), 4.19 - 3.97 (m, 3H, H-6a and b, H-2), 2.00, 1.97, 1.93, 1.79 (4 × s, 3H, OAc) ¹³C NMR (400 MHz DMSO): 81.45 (C-1) 75.51 (C-5) 73.57 (C-4) 62.23 (C-6) 52.05 (C-2) 21.24 (OAc)

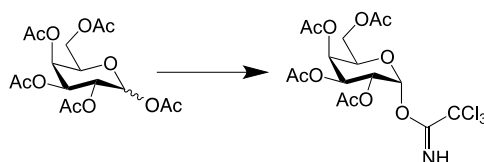
2-N-Acetamido-3,4,6-tri-O-acetyl-1-chloro-2-deoxy-α-D-glucopyranoside (11)



Glucosamine (20g, 90.4mM, 1equiv) was added to acetyl chloride (58.1mL, 814mM, 9 equiv) over 3 minutes under an argon atmosphere. The

suspension was stirred for 2 days at room temperature, a dark red color developed. 100mL of chloroform was added to the suspension, which was subsequently poured over ice (100g) and water (25mL). The organic layer was then transferred into 50g of ice to which 125mL of saturated NaHCO_3 was added. The organic layer was dried with MgSO_4 , filtered and the solvents evaporated. This was then dissolved in the minimum amount of chloroform and recrystallised with diethyl ether. The solid separated by Buchner filtration and washed with cold diethyl ether to the product white crystals (33.10g, 91%). **Rf** (100% EtOAc = 0.48). **[α] $_{\text{D}}^{25}$** 85.5 (c 0.5 CHCl_3). **LR-ESI-TOF-MS** **m/z**: = 388.05 $[\text{M} + \text{Na}]^+$, 753.14 $[2\text{M} + \text{Na}]^+$ **^1H NMR** (400MHz CDCl_3) δ (ppm): 6.18 (d, $J = 3.72\text{Hz}$, 1H, H-1), 5.86 (d, 1H, NH), 5.32 (dd, $J=9.47$ $J= 10.60$, 1H, H-3), 5.21 (m, 1H, H-4), 4.53 (ddd $J= 10.6$, $J= 8.76$, $J= 3.69$, 1H, H-2), 4.27 (m, 2H, H-6a, H-5), 4.15 (m, 1H, H-6b), 2.10, 2.05 and 1.99 (4 $\times$ s, 3H, OAc). **^{13}C NMR** (400MHz CDCl_3): 93.62 (C1) 70.87 (C5) 70.10 (C3) 66.92 (C4) 61.77 (C6) 53.46 (C2) 23.06, 20.66 and 20.53 (4 $\times$ OAc).

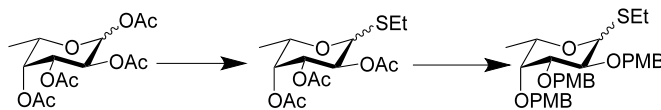
2,3,4,6-Tetra-O-acetyl- α -D galactopyranosyl trichloroacetimidate (10)



Peracetylated galactose (10g) was dissolved in 100mL of anhydrous DMF to which 2.83g (1.2 equiv 0.031 mol) of hydrazine was added and was left stirring at 50°C under N_2 for 1 hour. The solvents were then reduced to a third of the volume under reduced pressure. 50mL Ethyl acetate was next added and the organic layer was washed once with 25mL brine and three times with 25mL water and dried over MgSO_4 . The solvents were evaporated followed by two co-evaporations with toluene to afford 1-deacetylated fucose as a colorless oil (8.91g, 25.59mM, 99% yield). The oil was dissolved in 37mL anhydrous DCM to which was added trichloroacetonitrile (10.5mL 10 equiv, 0.114 mol) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (0.303mL 2.26mM 0.2 equiv). The reaction was left stirring under an argon atmosphere overnight. The

reaction was then concentrated down under reduced pressure and the dark oil was purified directly by flash chromatography (EtOAc:PE 3:1) to yield the product as an amorphous white solid (6.96g, 17.12 mM, 78% yield). **R_f**=0.65 (EtOAc) **[α]_D²⁵** α +108.8 (c 0.5, CHCl₃) **LR-ESI-TOF-MS m/z**: [M+Na]⁺=515.99; [2M+Na]⁺ =1008.97. **R_f** (EtOAc:PE 3:1) =0.63 **¹H NMR** (CDCl₃ 400MHz) δ (ppm) α -anomer: 8.67 (s, 1H, NH), 6.60 (d, J=3.44, 1H, H-1), 5.56 (d, J=3.08, 1H, H-2), 5.45-5.35 (dd, J=10.85, J=3.08, 1H, H-3), 5.41-5.38 (dd J=10.85, J=3.44, 1H, H-2), 4.44 (t, J=6.6Hz, 1H, H-5), 4.19-4.06 (m, 2H, H-6), 2.17 (s, 3H, AcO) 2.04, 2.03, 2.02 (s, 9H, AcO) **¹³C NMR** (CDCl₃ 400MHz): 170.29, 170.09, 170.07, 169.97 (4xC=O), 160.93(C=NH), 93.50 (C1), 68.97 (C5), 67.49(C3), 67.34 (C2), 66.88 (C4), 61.23(C6), 20.65 (C(O)CH₃), **M.P** 103-109°C

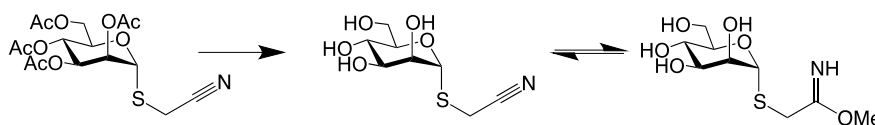
Ethyl 2,3,4-tri-*O*-*para*-methoxybenzyl-1-thio-β-L-fucopyranoside (9) and Ethyl 2,3,4-tri-*O*-*para*-methoxybenzyl-1-thio-α-L-fucopyranoside (9)



L-Fucose (10g 60.9mM) dissolved in pyridine (100mL) and acetic anhydride (200mL) and stirred for 1 hour at 100°C. The solvents were then evaporated to afford 2 (20.20g) which was dissolved in anhydrous DCM (33mL/mM) and was cooled to 0°C. Ethanethiol (9mL, 121.8mM, 2 equiv) and BF₃.OEt₂ (11.3mL, 91.35mM, 1.5 equiv) was added and the solution was stirred at room temperature over night. The reaction was worked up with saturated NaHCO₃ (2 x 50mL), water (2x50mL) and brine (50mL) and subsequently dried over MgSO₄ followed by evaporation of the solvent to afford 3 (19.76g) was dissolved in anhydrous MeOH (120mL/60mL) and NaOMe (3.52g 653.07mM, 1.1 equiv) was then added and the solution was stirred over night. The reaction was neutralized using Dowex resin, followed by filtration over Celite. The solvents were then evaporated to yield the deacylated thioglycoside (7.89g 37.88mM). This was dissolved in anhydrous DMF (2.5mL/mM) and was cooled to 0°C, NaH was then added (9.09g, 227.29 mM, 6 equiv) and the solution was stirred for 2 hours at 0°C. PMBCl

(18.49mL, 136.37 mM, 3.6 equiv) was added and the solution was stirred overnight before the addition of MeOH (50mL) and the evaporations of the solvents. The residue was taken up with EtOAc and the organic layer was washed with H₂O (3x100mL) and brine (1x 100mL) and dried over MgSO₄. The product was a orange oil and was purified by flash chromatography (PE:EtOAc, 9:1, 85:15, 8:2, 6:4) to afford the PMB protected α and β - anomers (20.49g 95% crude yield 1:3.6 α : β). **Rf** (PE:EtOAc 1:2)= 0.74(α) and 0.53 (β) **[α]_D²⁵** α -101.2 (c 0.5, CHCl₃), β +1.2 (c 0.5, CHCl₃) **LR-ESI-TOF-MS m/z**: [M+Na]⁺=591.30 **¹H NMR** (CDCl₃ 400MHz) δ (ppm): α -anomer: 7.27 (m 7H, HAr), 6.89 (m 6H HAr), 5.41 (d J= 5.52Hz, 1H, H-1) , 4.91 (d J=11.36Hz, 1H, CH₂bz), 4.79 (d, J=11.41Hz, 2H, CH₂Bz), 4.69 (m, 2H, CH₂Bz) 4.25 (m, 3H, H-2, H-5) 3.74 (m, 1H, H-3), 3.57 (m, 1H, H-4), 2.55 (m, 2H, SCH₂), 1.26 (t, J=6.48Hz, 3H SCH₂CH₃), 1.07 (d, J=6.48Hz, CH₃) **¹³C NMR** (CDCl₃ 400MHz): 131.04-129.13 (Ar), 113 (Ar) 83.50 (C-1), 79.57 (C-3), 76.70 (C-4), 75.67 (C-2), 74.33 (CH₂Bz), 73.08 (CH₂Bz) 72.01 (CH₂Bz), 66.59 (C-5), 23.70 (SCH₂CH₃), 16.57 (C-6), 14.19 (SCH₂CH₃). **¹H-NMR** (CDCl₃ 400MHz) δ (ppm) β -anomer: 7.37 (m, 8H, HAr), 6.91 (m, 6H, HAr), 4.94 (d, J=11.4Hz, 1H, CH₂bz), 4.84 (d, J=11.05Hz, 1H, CH₂Bz), 4.82 (d, J=11.93Hz, 1H, CH₂Bz), 5.41 (d J=9.64Hz, 1H, H-1), 3.79 (m, 1H, H-2) 3.57 (m, 2H, H-3, H-4) 3.52 (q, J= 6.32Hz, 1H, H-5), 2.76 (m, 2H, SCH₂CH₃) 1.31 (t, J=7.42Hz, 3H, SCH₂CH₃) 1.18 (d, J=6.32Hz, 3H, CH₃) **¹³C NMR** (CDCl₃ 400MHz): 159.25 (Ar) 130.85-125.30 (Ar), 113.81-113.49 (Ar) 84.98 (C-1), 85.33 (C-3), 78.08 (C-2), 75.85 (CH₂Bz), 75.31 (C-4), 74.52 (CH₂Bz), 73.98 (C-5), 72.54 (CH₂Bz), 55.28 (OCH₃), 24.67 (SCH₂CH₃), 17.23 (C-6), 14.99 (SCH₂CH₃).

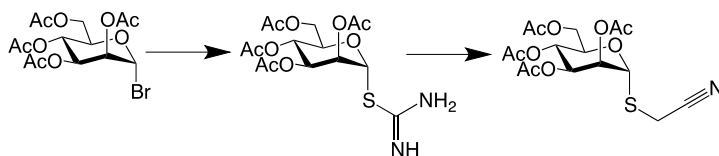
1-thio-(S-cyanomethyl)- α -D-mannopyranoside (7) and 1-thio-(S-2-imino-2-methoxyethyl)- α -D- mannopyranoside (8)



100mg of **6** was suspended in 1mL of MeOH to which 57 μ L of 25% NaOMe in MeOH (1 equiv 0.24mM). After 16 hours then solvent was evaporated by

nitrogen flow and followed by vacuum to yield a white amorphous solid. The product was store under an inert atmosphere at -20°C . **Rf** (9:1 acetone:water)= 0.7 (cyanomethyl thiol), 0.4 (IME) . **LR-ESI-TOF-MS m/z** $[\text{M}+\text{Na}]^{+}=258.0$ (cyanomethyl thiol), $[\text{M}+\text{Na}]^{+}=291.0$ (IME), $[\alpha]_{\text{D}}^{25}+56.85$ (c, 0.250, CHCl_3), **^1H NMR** (CDCl_3 400MHz) δ (ppm): 5.33 (d, 1H, $J=0.94$, H-1 (7)), 5.09 (br s, 1H, H-1(8)), 3.84 (dd, 1H, $J=3.42$, $J=1.30$ H-2 (7)), 3.83 (m, 1H, H-1 (8)), 3.82-3.75 (m, 5H, H6a and b (7)(8), H-5 (7)), 3.69-3.49 (m, 7H, H-4 (7)(8), H-3 (7)(8)), **^{13}C NMR** (CDCl_3 400MHz): 84.71 (C-1 (7)(8)), 74.39 (C-3 (7)), 74.12 (C-3(8)), 71.88 (C-4 (8)), 71.8 (C-4(7), 71.72 (C-2 (8)), 71.37 (C-2(7)), 67.35 (C-5 (8)), 67.14 (C-5(7)), 61.23 (C-6(8)), 61.07 (C-6(7)). **M.P.** $108-109^{\circ}\text{C}$.

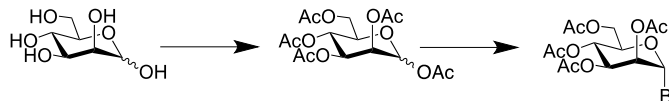
2,3,4,6- Tetra -O-acetyl -1-thio-(S-cyanomethyl)- α -D-mannopyranoside (6)



9.22g of **5** was and thiourea (2.2 equiv, 44mM, 3.77g) were suspended in anhydrous acetone (50mL) and heated at 80°C for 2 hours after which the acetone was evaporated to yield a white amorphous solid. **Rf** (EtOAc: PE 1:1)=0. **LR-ESI-TOF-MS m/z** $[\text{M}+\text{H}]^{+}=407.1$. The dry crude product, $\text{Na}_2\text{S}_2\text{O}_5$ (2.7 equiv, 59mM, 11.3g) and K_2CO_3 (1.5 equiv, 33mM, 4.56g) were then dissolved in 1:1 water:acetone 100mL before the addition of chloroacetonitrile (5 equiv, 110nM, 7mL). The suspension was stirred vigoursly to promote mixing of the two phases. After 1 hour the migratory of the acetone fraction was evaporated and the remaining was extracted with DCM (100mL). This organic layer was then washed with brine (x2) and H_2O . (6.4g 68% yield) **Rf** (EtOAc: PE 1:1)=0.4. **LR-ESI-TOF-MS m/z** $[\text{M}+\text{Na}]^{+}=426.1$ $[\alpha]_{\text{D}}^{25}+56.85$ (c, 0.250 CHCl_3) (Lit $[\alpha]_{\text{D}}^{22}+74.5$ (c, 1.0 CHCl_3)) ⁷³, **^1H NMR** (MeOD 400MHz) δ (ppm): 5.34 (d, 1H, $J=1.3\text{Hz}$, H-1), 5.31-5.29 (m, 1H, H-2), 5.26-5.23 (m, 1H, H-4), 5.15 (dd, $J=10.02$, $J=3.46$, H-3), 4.31-4.23 (m, 2H, H-6a, H-5), 4.11-4.01 (m, 1H, H6b), 3.43 (d, 1H, $J=17.16$, SCH_2CN), 3.28 (d, 1H, $J=17.16$, SCH_2CN), 2.12, 2.04, 1.99, 1.93 (4xs 12H, 4xOAc). **^{13}C**

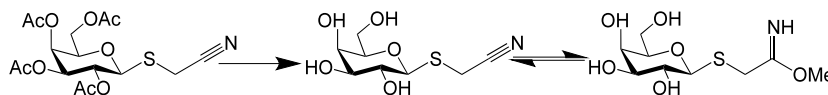
NMR (MeOD 400MHz) δ (ppm): 115.6 (CN), 82.4 (C1), 69.9 (C2), 69.6 (C5), 69.2 (C4), 65.8 (C3), 62.0 (C6), 20.8, 20.7, 20.6, 20.6 (OAc).

2,3,4,6-Tetra-*O*-acetyl- α -D-mannopyranosyl bromide (5)



5g of mannose was suspended in pyridine (100mL) and acetic anhydride (200mL) before the addition of dimethylaminopyridine DMAP (0.2 equiv. 680mg). After 2 hours 100mL of DCM was added and the organic layer was then washed with aqueous 5% CuSO₄, HCl and H₂O and then evaporated to yellow oil **Rf** (EtOAc: PE 1:1)=0.7 **LR-ESI-TOF-MS** m/z [M+Na]⁺=413.1. This crude product was then dissolved in HBr (33% in acetic anhydride) (40mL) and acetic anhydride (10mL), after 2 hours the product was extracted with DCM. The organic layer was washed with ice cold aqueous NaHCO₃, brine and H₂O, before drying over MgSO₄ and evaporation to a yellow oil (9.22g 78% yield) **Rf** (EtOAc: PE 1:1)=0.85, $[\alpha]_D^{25} +107$, (c, 1, in CHCl₃) (Lit $[\alpha]_D^{22} + 124$ (c, 1.0 CHCl₃)) ⁷⁶**¹H NMR** (CDCl₃ 400MHz) δ (ppm) α -anomer: 6.23 (d, J= 1.08Hz, 1H, H-1,), 5.66-5.63 (dd, J=10.16Hz, J=3.44Hz, 1H, H-3), 5.38-5.37 (dd, J=3.38Hz, J=1.58Hz, 1H, H-2,), 5.33-5.27 (dd, J=10.18Hz, 1H, H-4,), 4.28-4.4 (m, 1H, H6a), 4.17-4.13 (m, 1H, H-5), 4.08-4.05 (m, 1Hm H6b), 2.11 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.95 (s, 3H, OAc). **M.P.** 133°C (Lit. 130-131°C ⁷³)

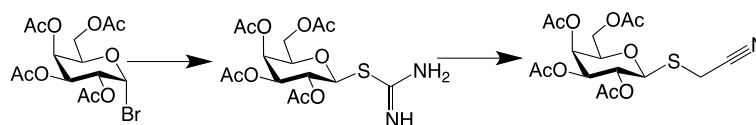
1-thio-(S-cyanomethyl)- β -D-mannopyranoside (3) and 1-thio-(S-2-imino-2-methoxyethyl)- β -D-galactopyranoside (4)



100mg of **2** was suspended in 1mL of MeOH to which 57 μ L of 25% NaOMe in MeOH (1 equiv 0.24mM). After 16 hours then solvent was evaporated by nitrogen flow and followed by vacuum to yield a white gum. The product was store under an inert atmosphere at -20°C. **Rf** (9:1 acetone:water)= 0.8 (cyanomethyl thiol), 0.75 (IME) . **LR-ESI-TOF-MS** m/z [M+Na]⁺=258.0

(cyanomethyl thiol), $[M+Na]^+ = 291.0$. $^1\text{H NMR}$ (MeOD 400MHz) δ ppm: 4.57 (d, 1H, $J=9.92\text{Hz}$, H-1,(3)), 4.22 (d, 1H, $J=9.54\text{Hz}$, H-1,(4)), 3.94. (m, 2H, H-4 (3)(4)), 3.84-3.79 (m, 4H, H-6a and b, (3)(4)), 3.69-3.47 (m, 6H, H-2 (3)(4), H-3(3)(4), H-5 (3)(4)) $^{13}\text{C NMR}$ (MeOD 400MHz) δ (ppm): 85.88 (C-1 (4)), 84.68 (C-1 (3)), 79.6 (C-2 (4)), 79.42 (C-2(3)), 75.02 (C-5 (4)), 74.99 (C-5(3)), 70.17(C-3 (4)), 70.12(C-3(3)), 69.30(C-4 (4)), 69.21 (C-4 (3)), 61.36 (C-6 (4)), 61.24 C-6 (3)).

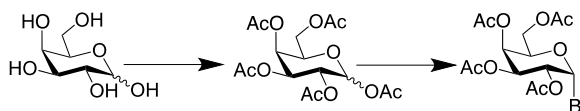
2,3,4,6- Tetra -O-acetyl -1-thio-(S-cyanomethyl)- β -D-mannopyranoside (2)



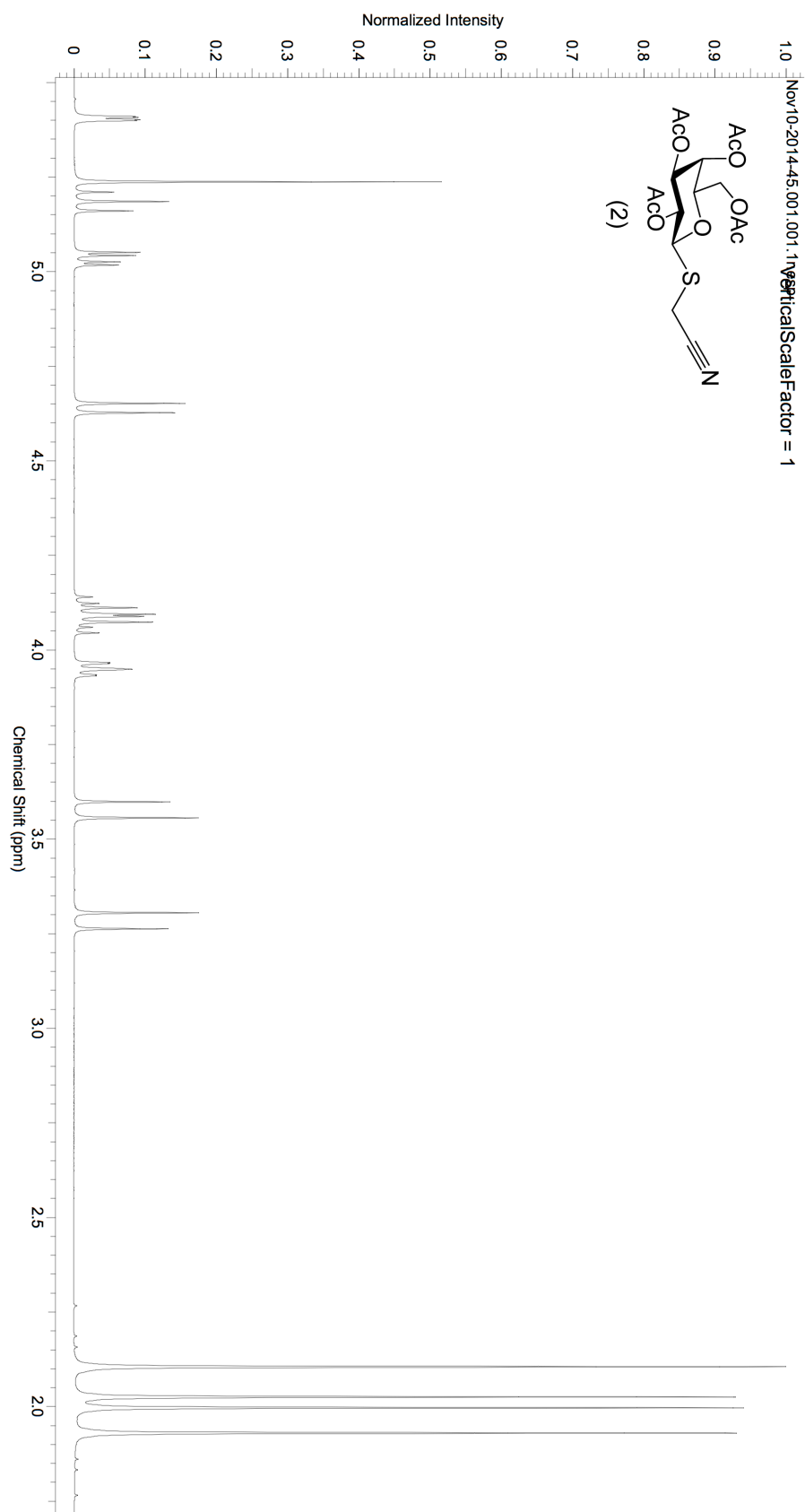
7.38g of **1** and thiourea (2.2 equiv, 36mM, 2.73g) were suspended in anhydrous acetone (50mL) and heated at 80°C for 2 hours after which the acetone was evaporated to yield a white amorphous solid. **Rf** (EtOAc: PE 1:1)=0. **LR-ESI-TOF-MS** m/z $[M+H]^+ = 407.1$. The dry crude product, $\text{Na}_2\text{S}_2\text{O}_5$ (2.7 equiv, 47mM, 9.24g) and K_2CO_3 (1.5 equiv, 27mM, 3.73g) were then dissolved in 1:1 water:acetone 100mL before the addition of chloroacetonitrile (5 equiv, 98mM, 5.7mL). The suspension was stirred vigorously to promote mixing of the two phases. After 1 hour the majority of the acetone fraction was evaporated and the remaining was extracted with DCM (100mL). This organic layer was then washed with brine (x2) and H_2O . (2.8g 30% yield) **Rf** (EtOAc: PE 1:1)=0.28. $[\alpha]_{\text{D}}^{25} -51.10$ (c, 0.125, CHCl_3) (Lit $[\alpha]_{\text{D}}^{22} -37.00$ (c, 1.0 CHCl_3)) ⁷³, **LR-ESI-TOF-MS** m/z $[M+Na]^+ = 426.1$. $^1\text{H NMR}$ (CDCl_3 400MHz) δ ppm : 5.41 (dd, 1H, $J= 3.30\text{Hz}$, $J=0.90\text{Hz}$, H-4), 5.18 (dd, 1H, $J=9.94\text{Hz}$, $J= 9.95\text{Hz}$, H-2), 5.02 (dd, 1H, $J=10.00\text{Hz}$, $J=3.32\text{Hz}$, H-3), 4.65 (d, 1H, $J=9.88\text{Hz}$, H-1), 4.11-4.07 (m, 2H, H-6a and b), 3.96 (m, 1H, H-5), 3.59 (d, 1H, $J=16.97\text{Hz}$ SCH_2CN), 3.30 (d, 1H, $J=16.97\text{Hz}$ SCH_2CN), 2.10, 2.02, 1.99, 1.93 (4xs, 12H, 4xOAc). **NMR** (CDCl_3 400MHz) δ (ppm): 115.8 (CN), 82.3 (C1), 75.0 (C5), 71.5 (C3), 67.1 (C4), 66.7 (C2), 61.4 (C6), 20.7, 20.7, 20.6, 20.5 (OAc), 14.5 (SCH_2CN). $^{13}\text{C NMR}$ (CDCl_3 400MHz) δ (ppm): 115.79 (CN), 82.25 (C-1), 75.04 (C-5), 71.47 (C-3), 67.11 (C-

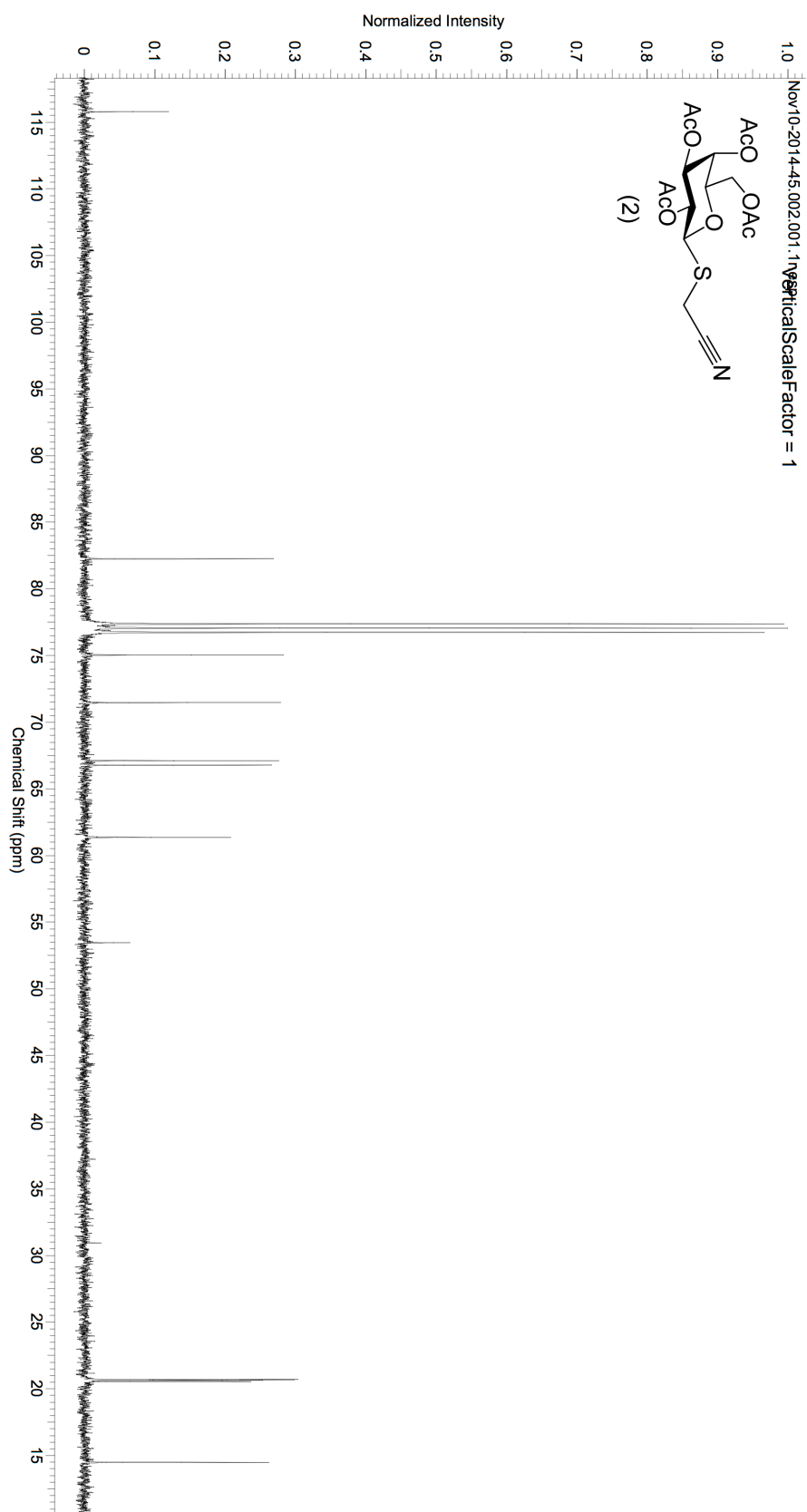
4), 66.77 (C-2), 61.36 (C-6), 20.69, 20.68, 20.64, 20.55 (OAc), 14.49 (SCH₂CN).
M.P. 97-102°C (Lit. 95-97°C⁷³)

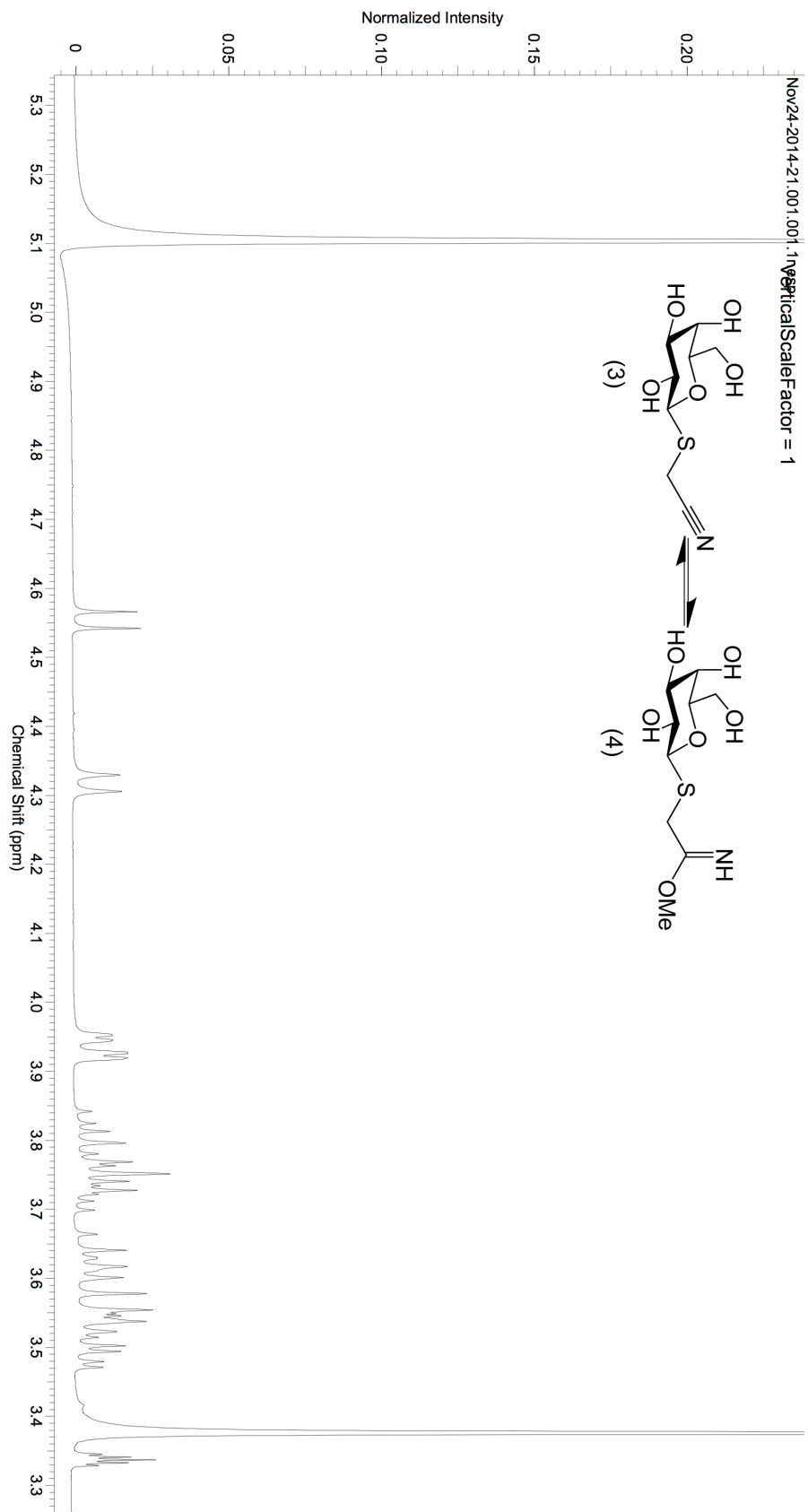
2,3,4,6-Tetra-O-acetyl- α -D-galactopyranosyl bromide (1)

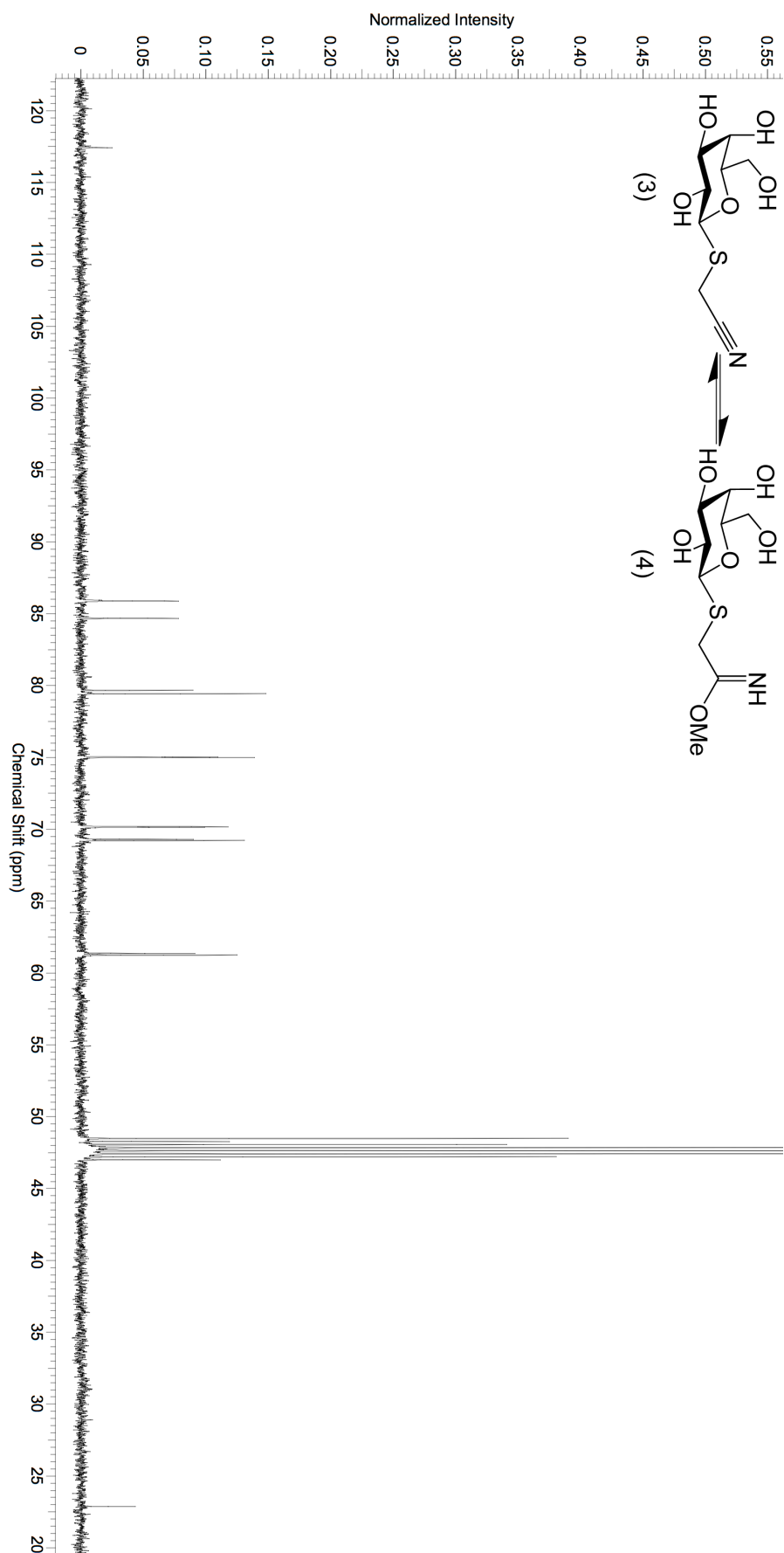


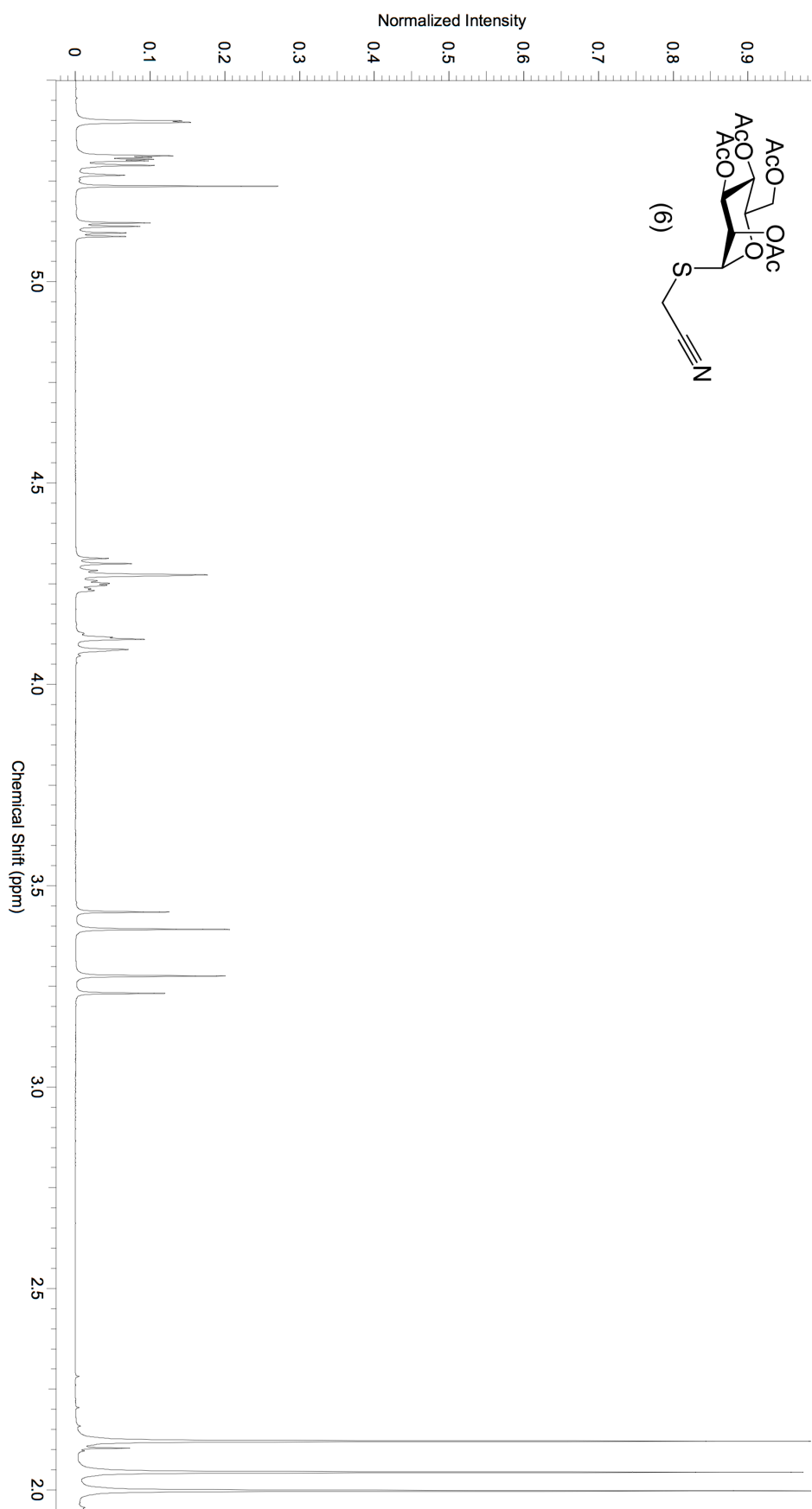
5g of galactose was suspended in pyridine (100mL) and acetic anhydride (200mL) before the addition of dimethylaminopyridine (DMAP)(0.2 equiv. 680mg). After 2 hours 100mL of DCM was added and the organic layer was then washed with aqueous 5% CuSO₄, HCl and H₂O and then evaporated to yellow oil **Rf** (EtOAc: PE 1:1)=0.8 **LR-ESI-TOF-MS** **m/z** [M+Na]⁺=413.1. This crude product was then dissolved in HBr (33% in acetic anhydride) (40mL) and acetic anhydride (10mL), after 2 hours the product was extracted with DCM. The organic layer was washed with ice cold aqueous NaHCO₃, brine and H₂O, before drying over MgSO₄ and evaporation to a yellow oil (7.28g 73% yield) **Rf** (EtOAc: PE 1:1)=0.9 [α]_D²⁵+201 (c, 1, CHCl₃), (Lit [α]_D²²+227 (c, 1.7 CHCl₃))⁷⁶, **¹H NMR** (CDCl₃ 400MHz) δ (ppm) α -anomer: 6.63 (d, J= 3.96Hz, 1H, H-1), 5.45-5.44 (dd, J=10.60Hz, J=3.24Hz, 1H, H-4), 5.35-5.31 (dd, J=10.64Hz, J=3.28Hz, 1H, H-3), 5.00-4.96 (dd, J=10.64Hz, J=3.96Hz, 1H, H-2), 4.43-4.0 (m, 1H, H-5), 4.14-4.02 (m, 2H, H-6a and b), 2.08 (s, 3H, OAc), 2.05 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.94 (s, 3H, OAc).

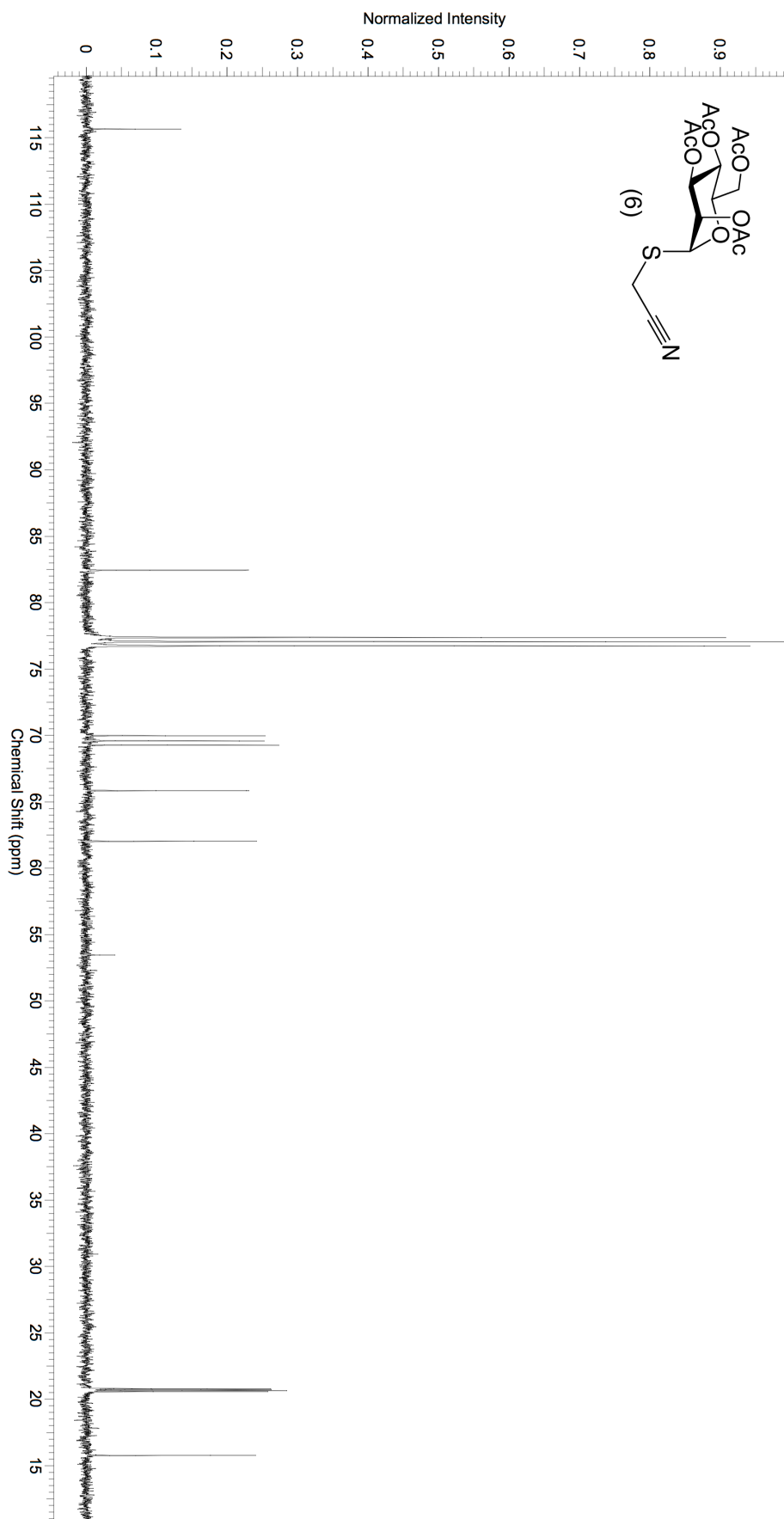


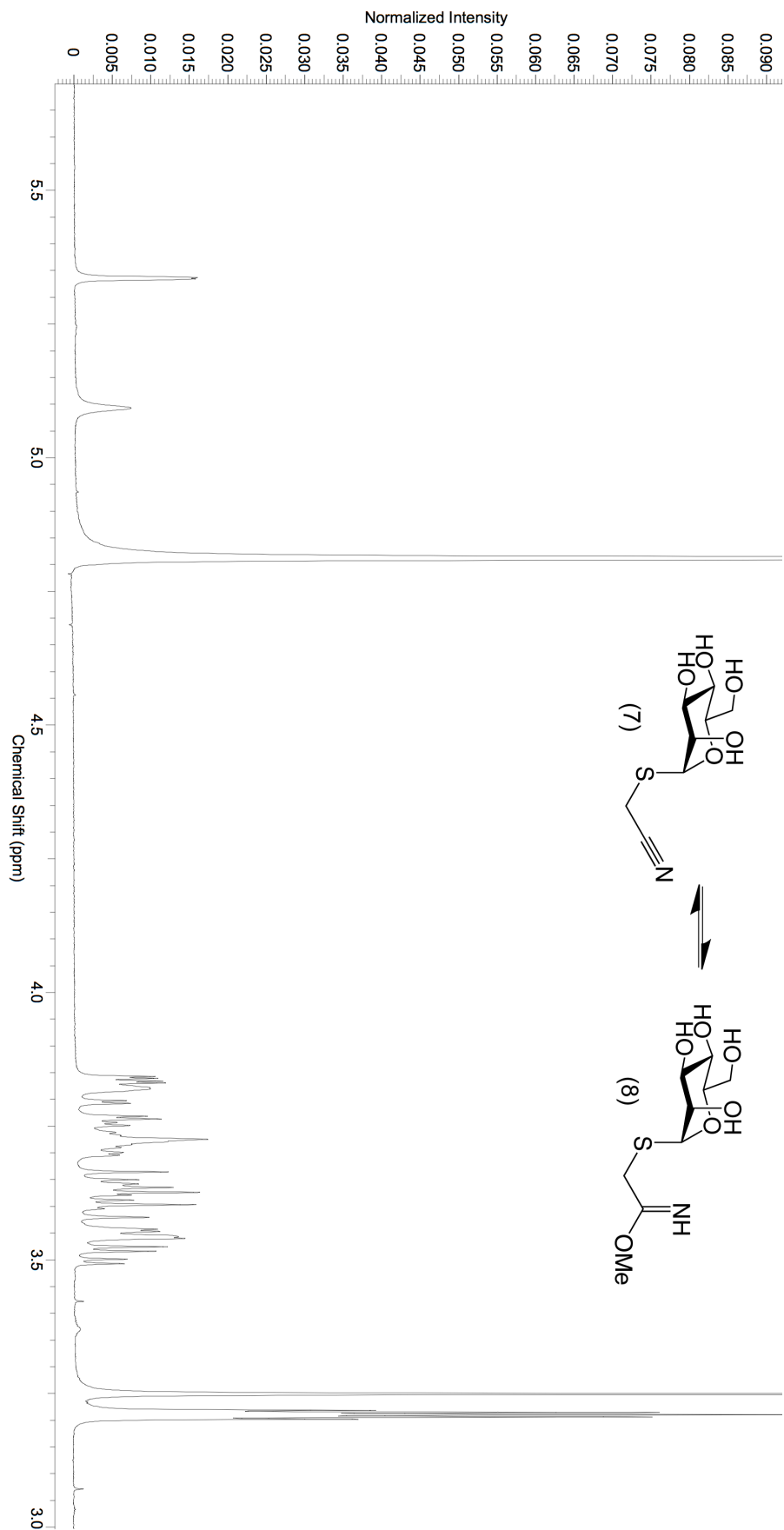


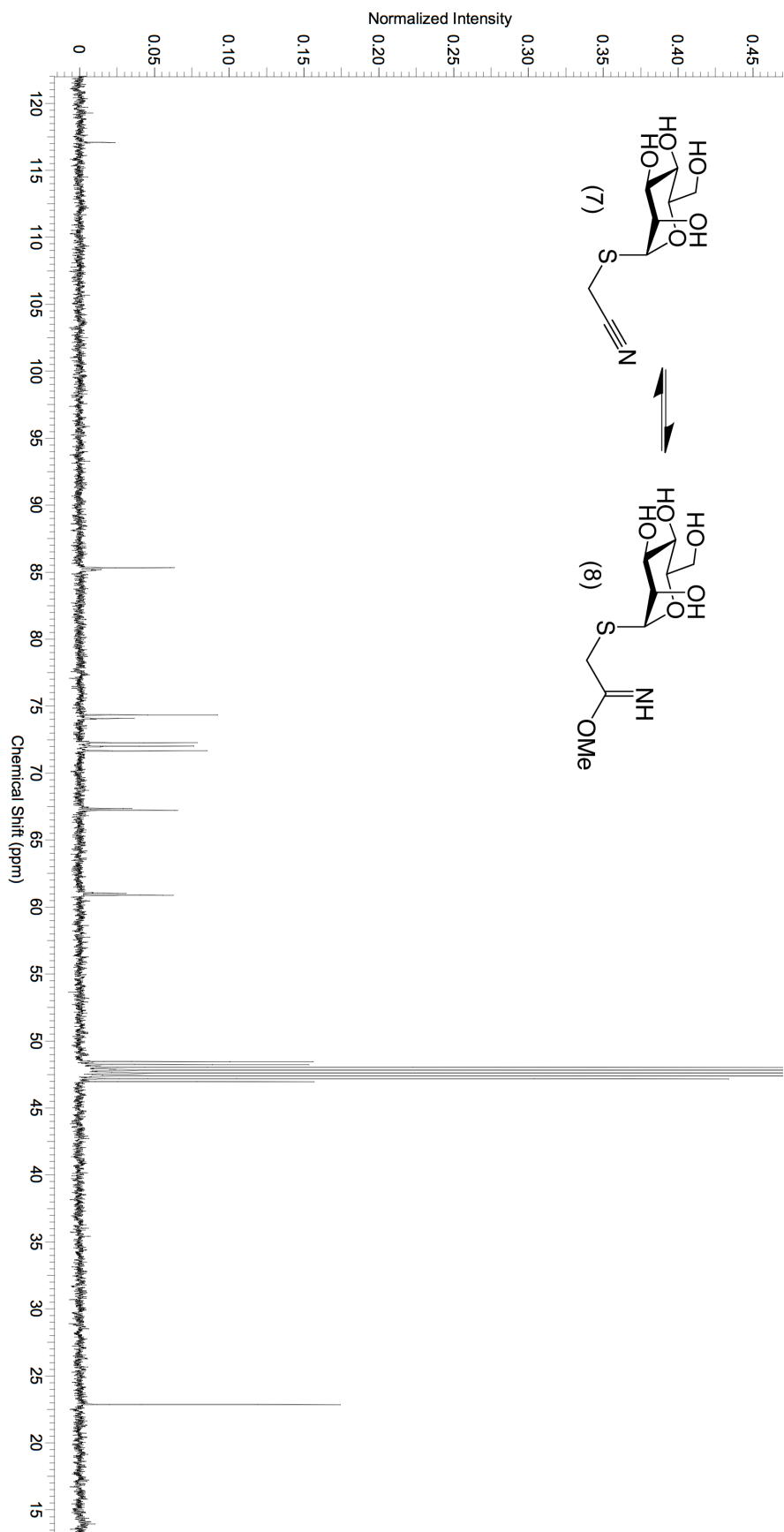


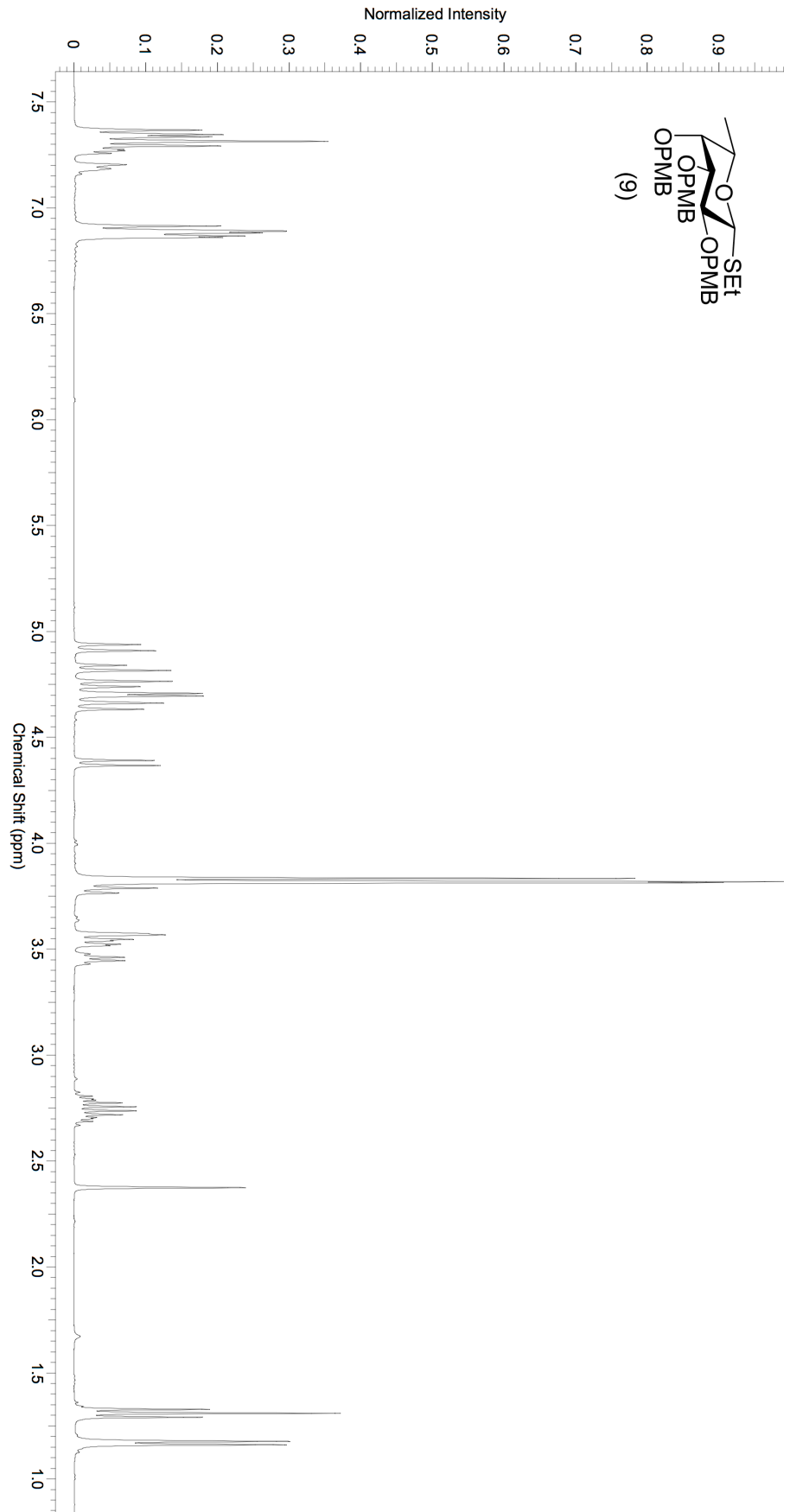


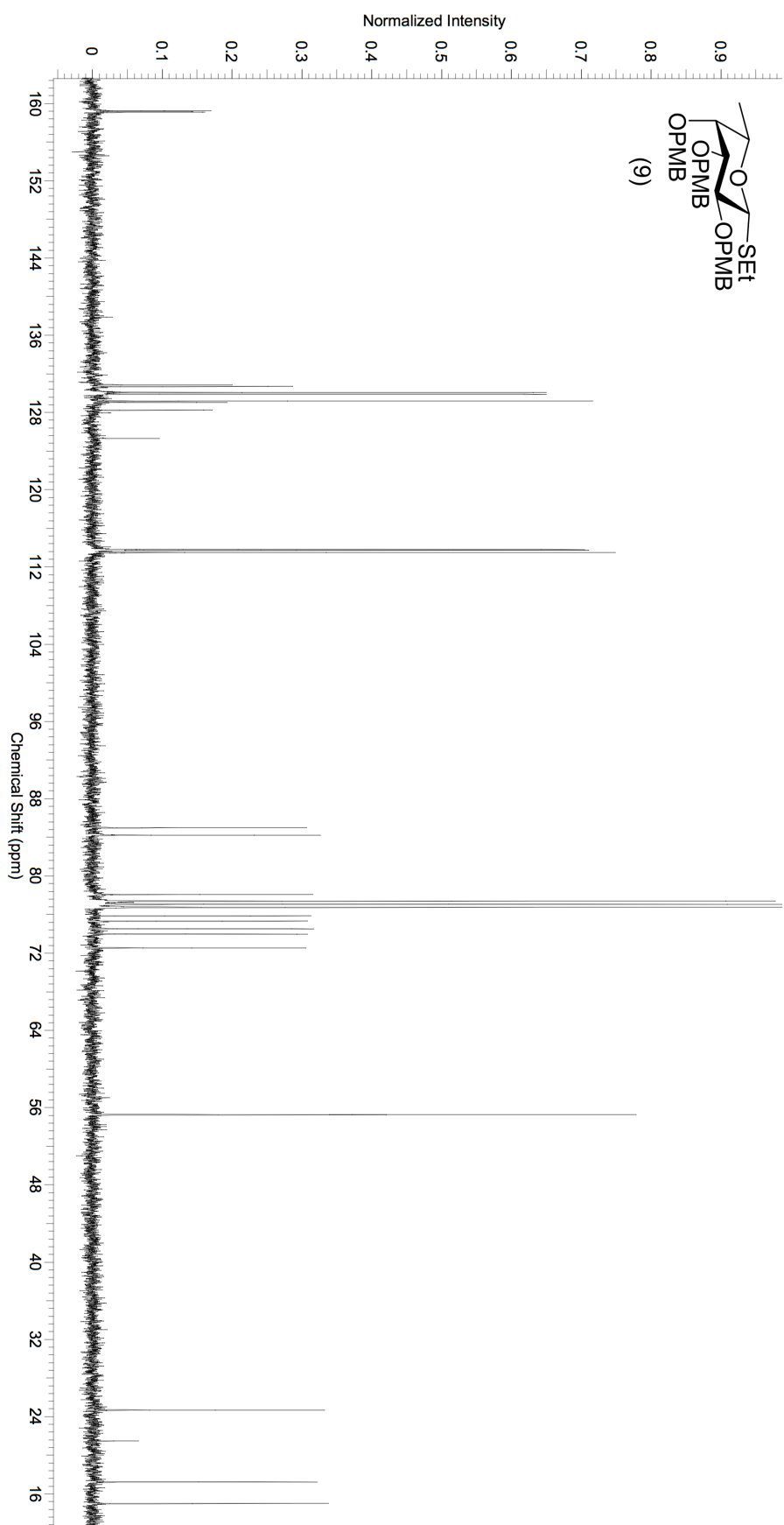


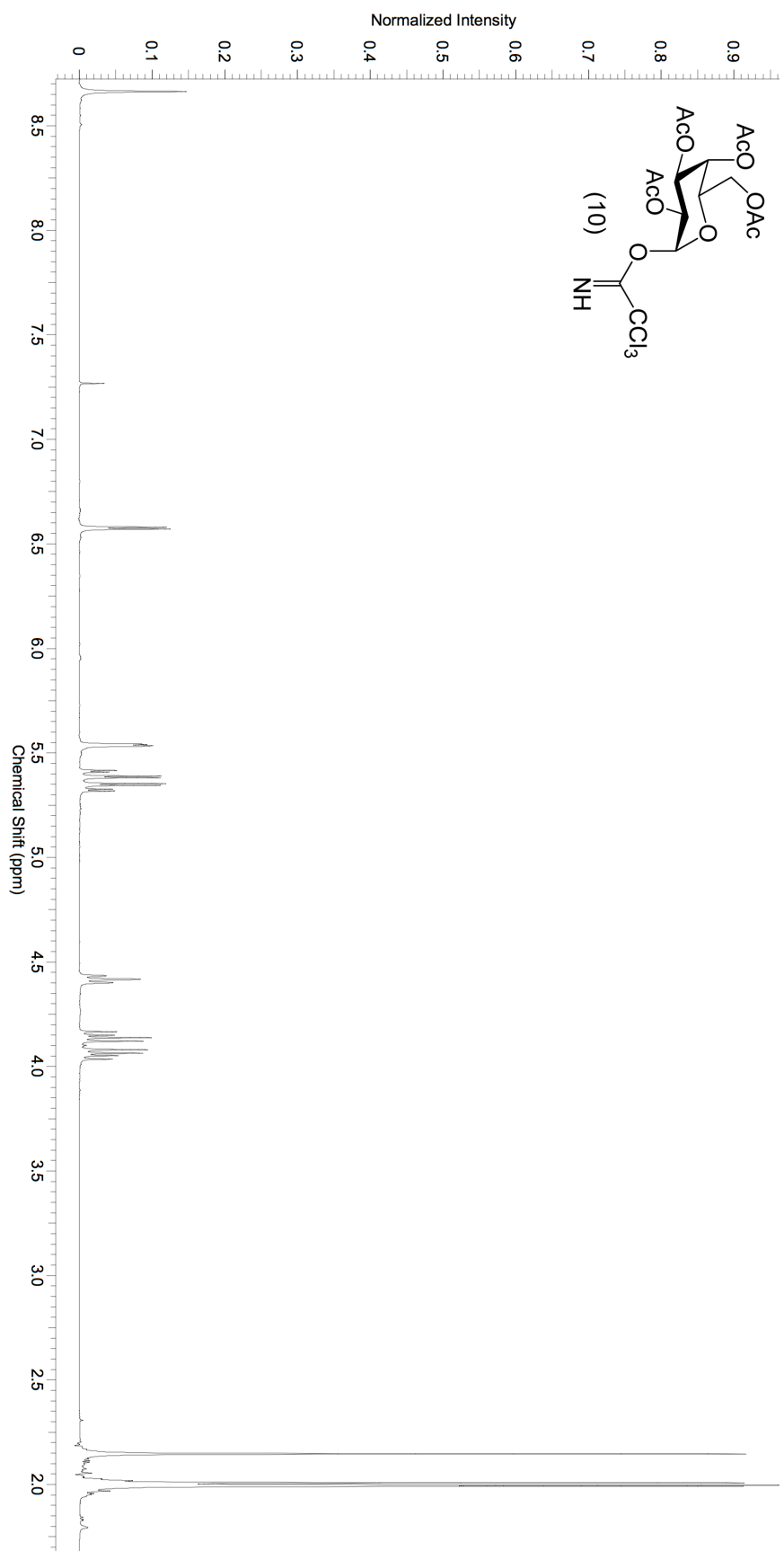


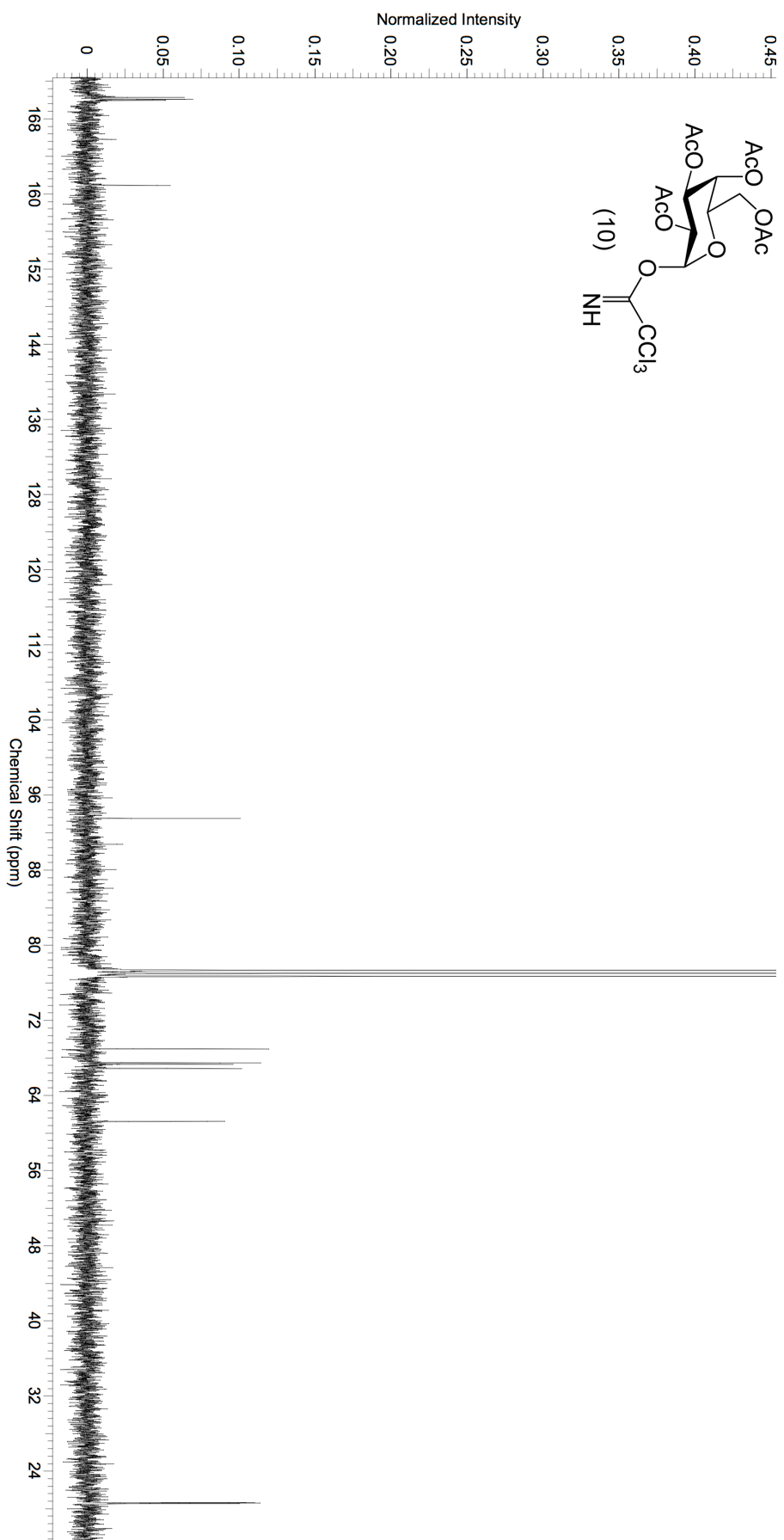


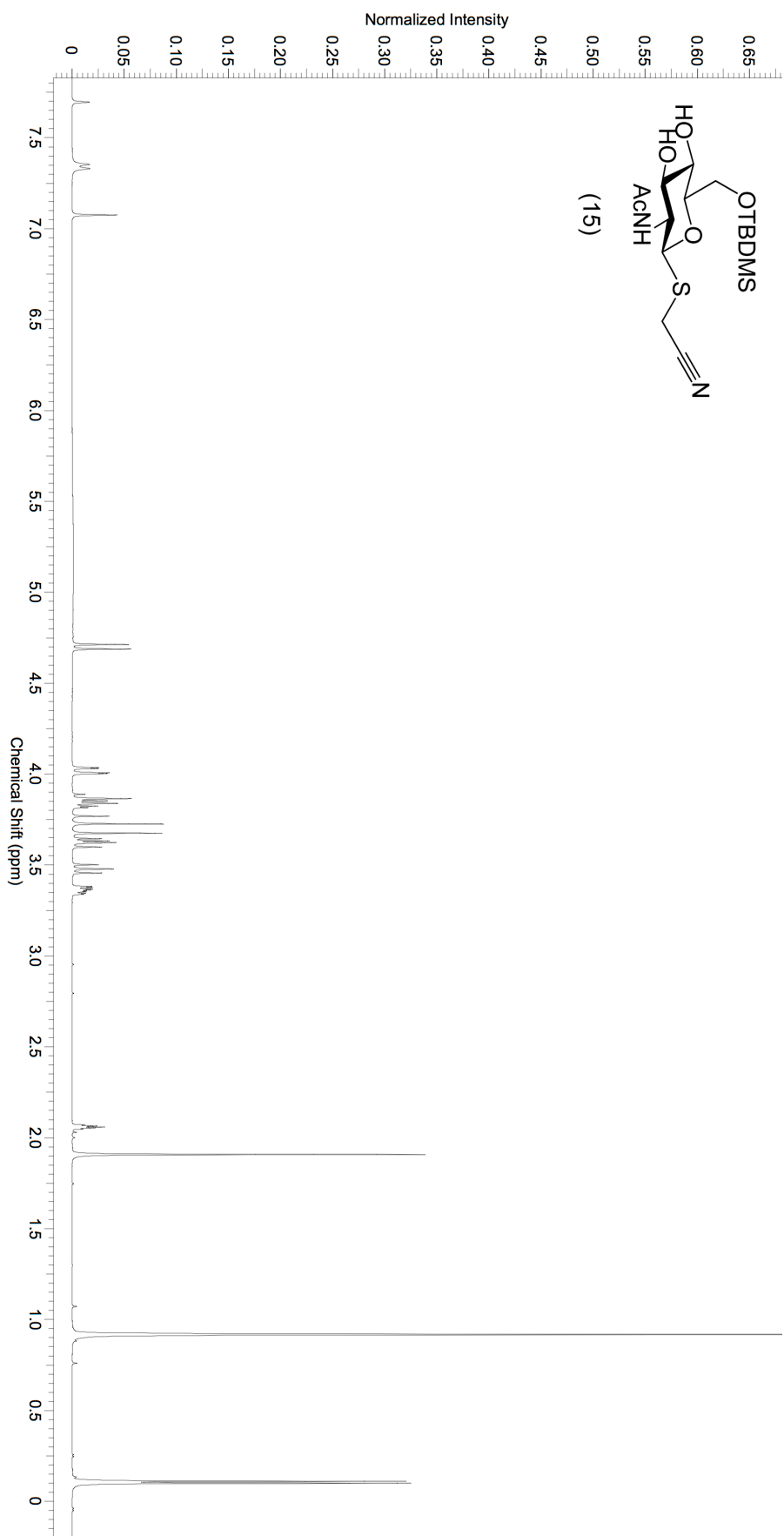


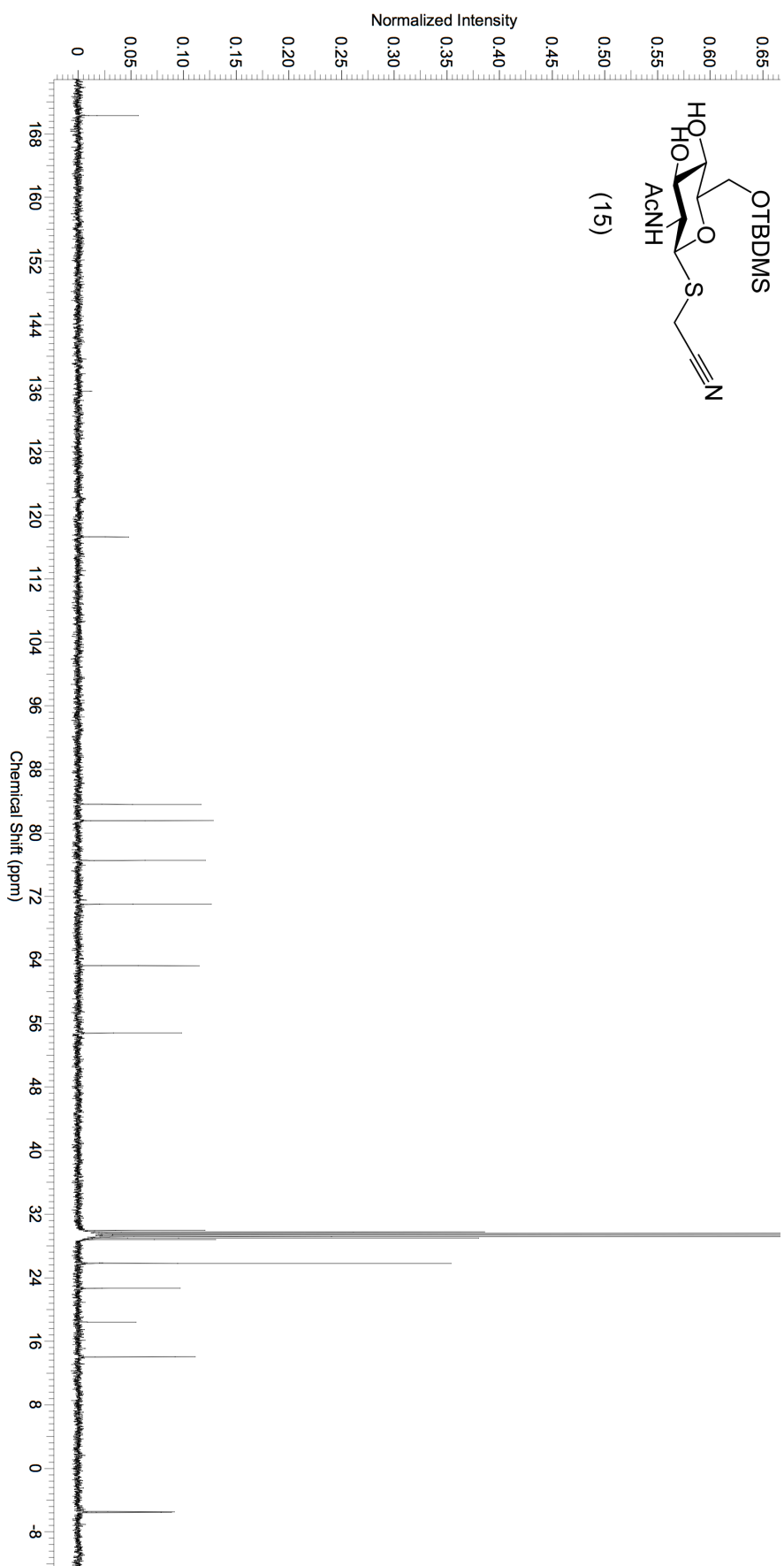


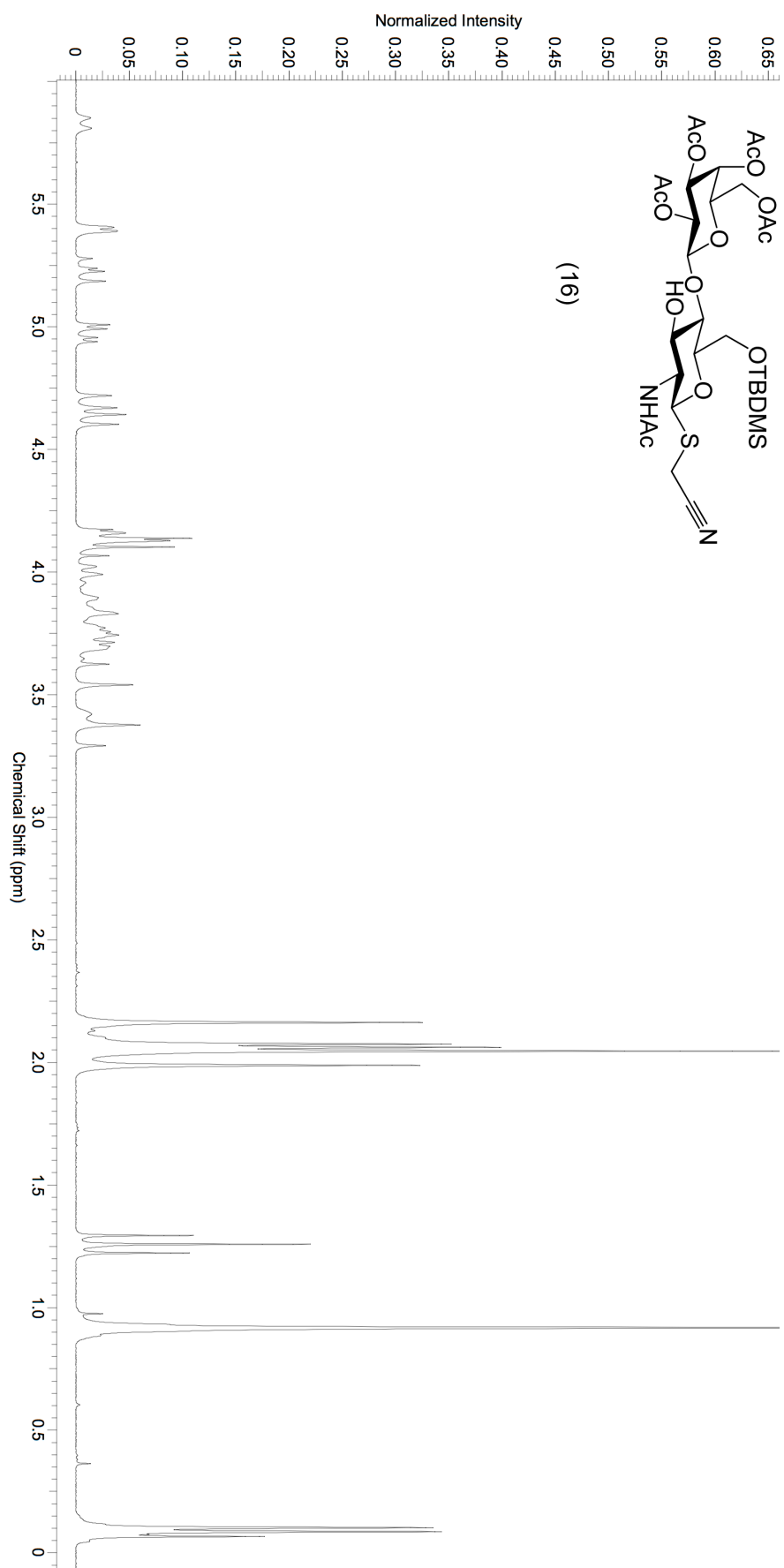


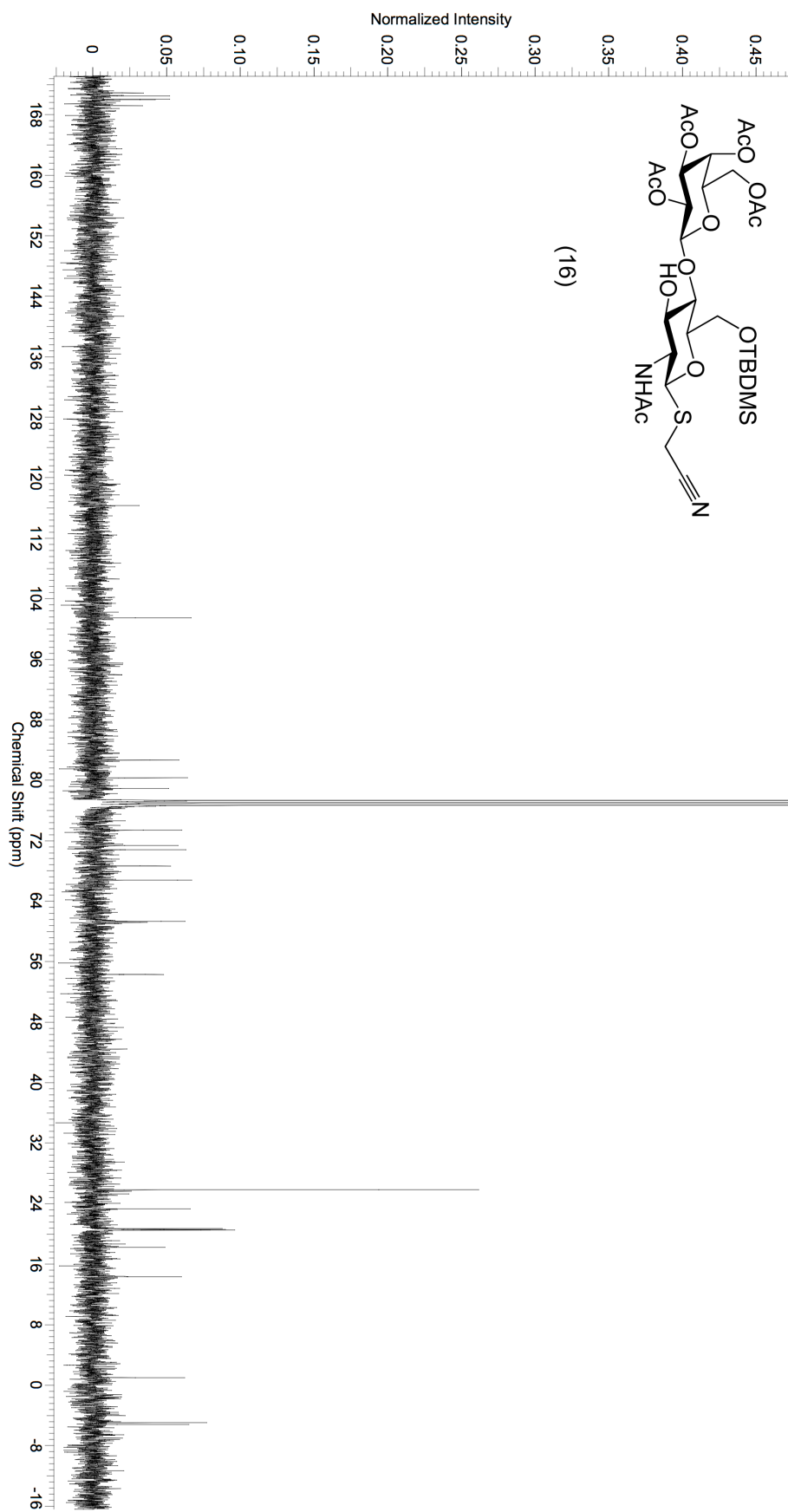


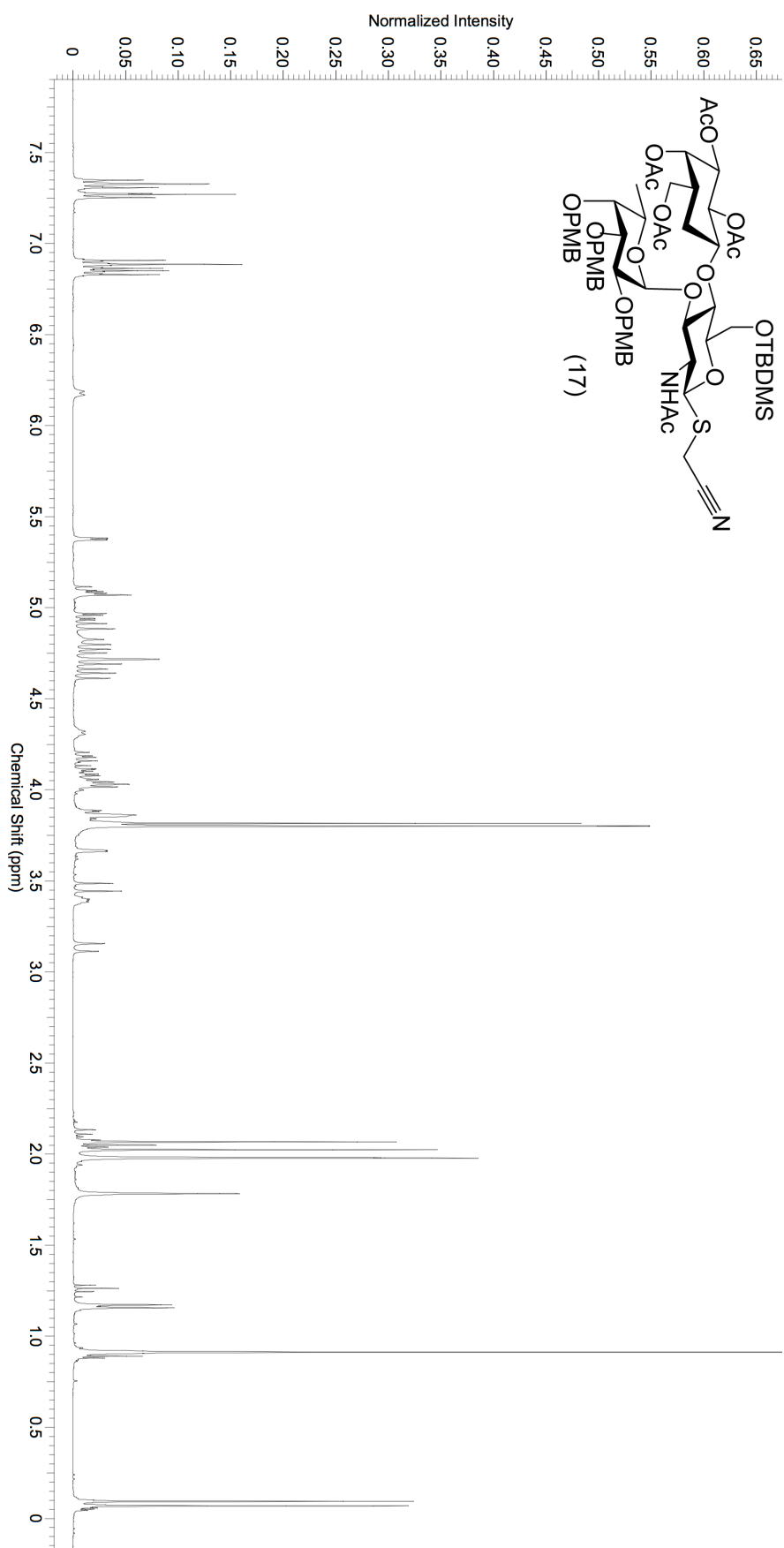


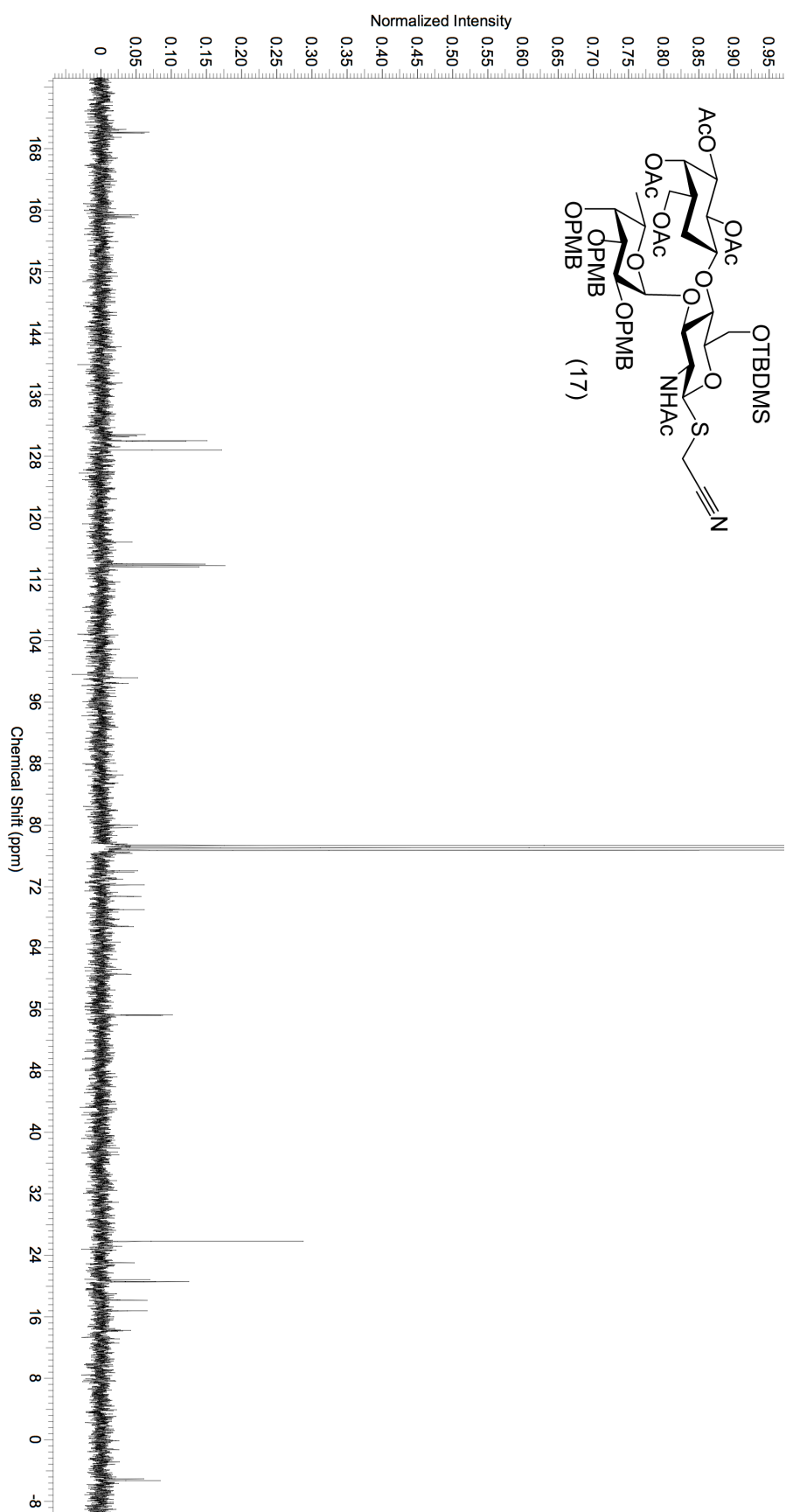


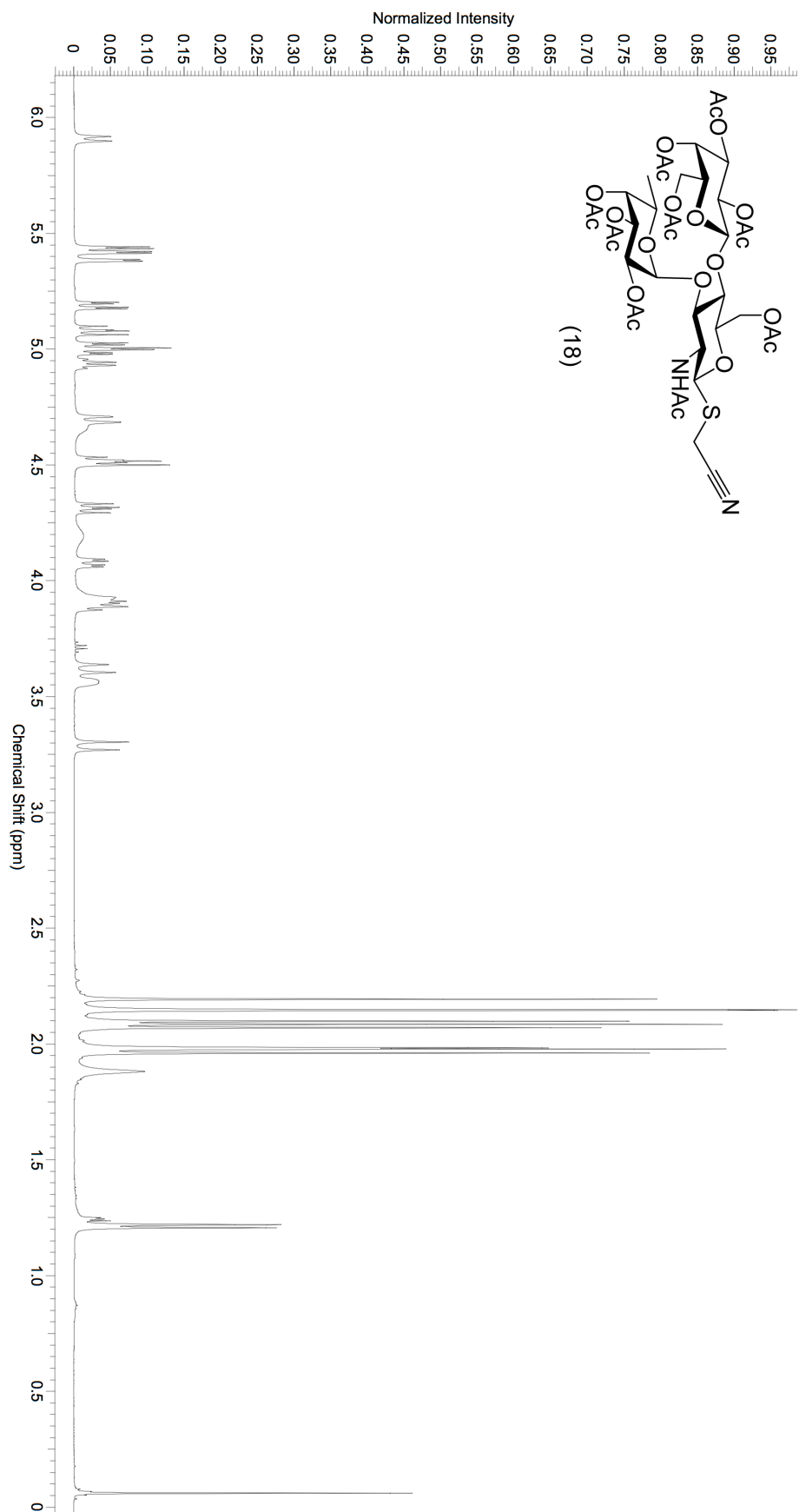


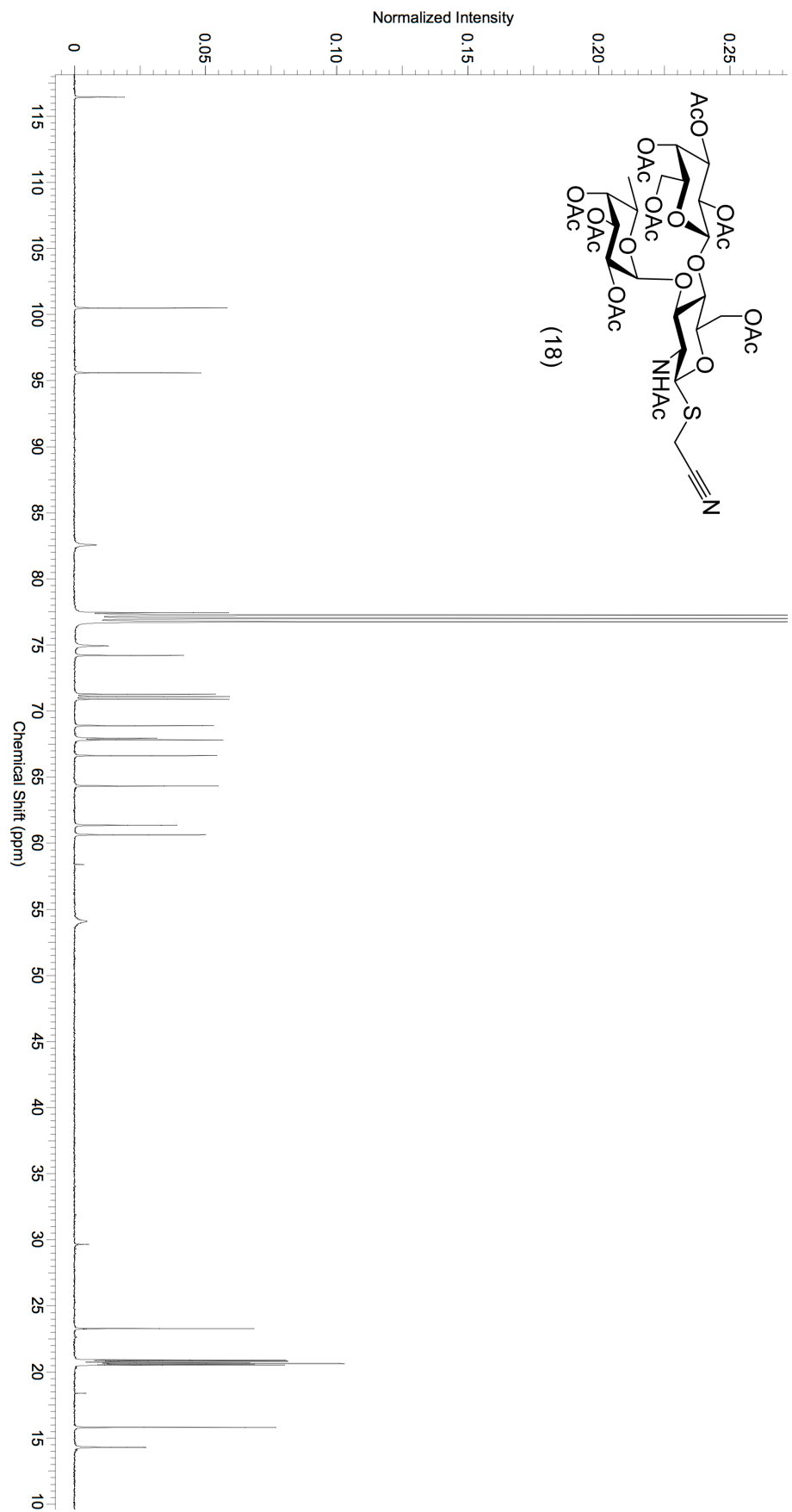


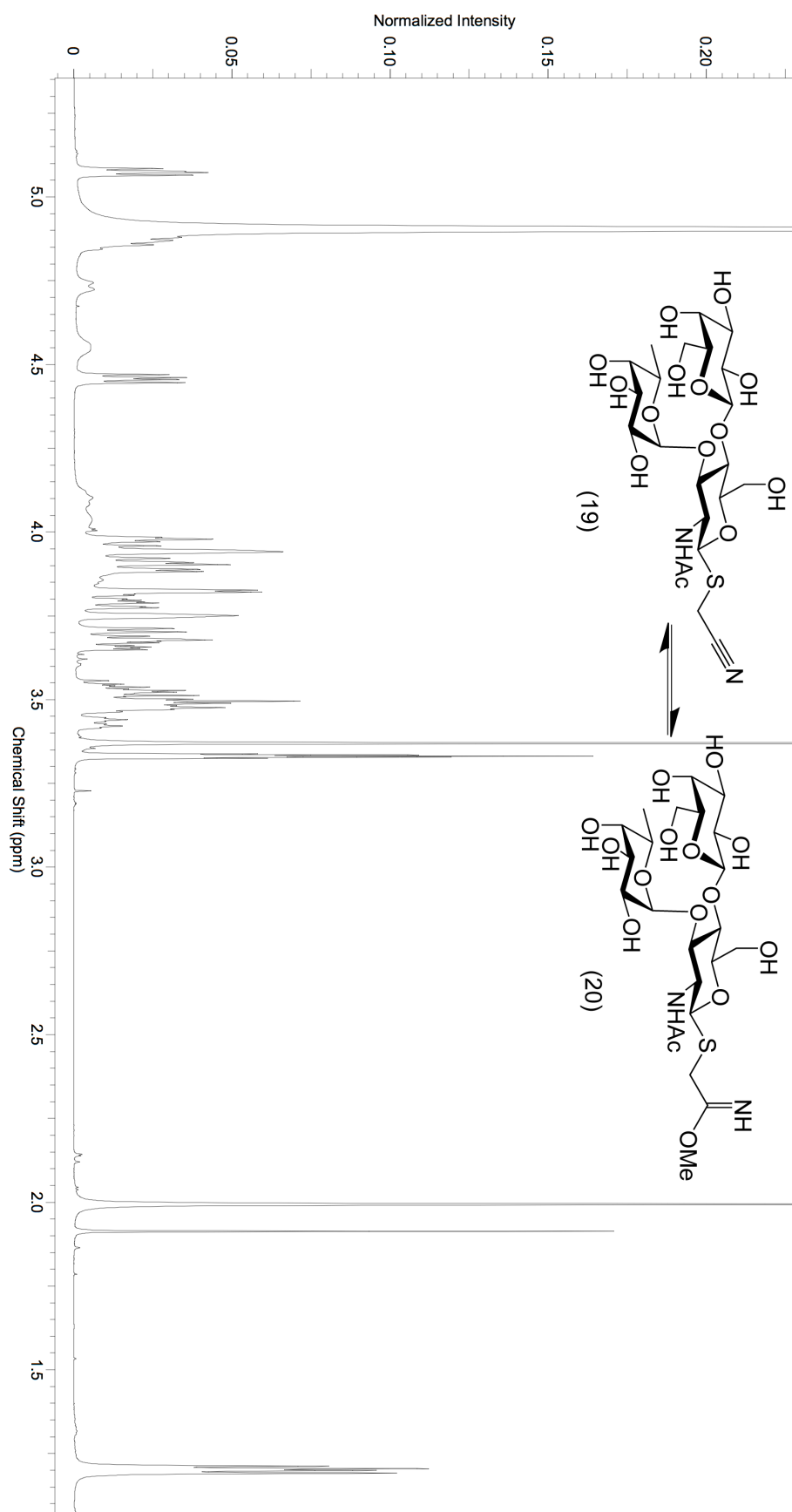


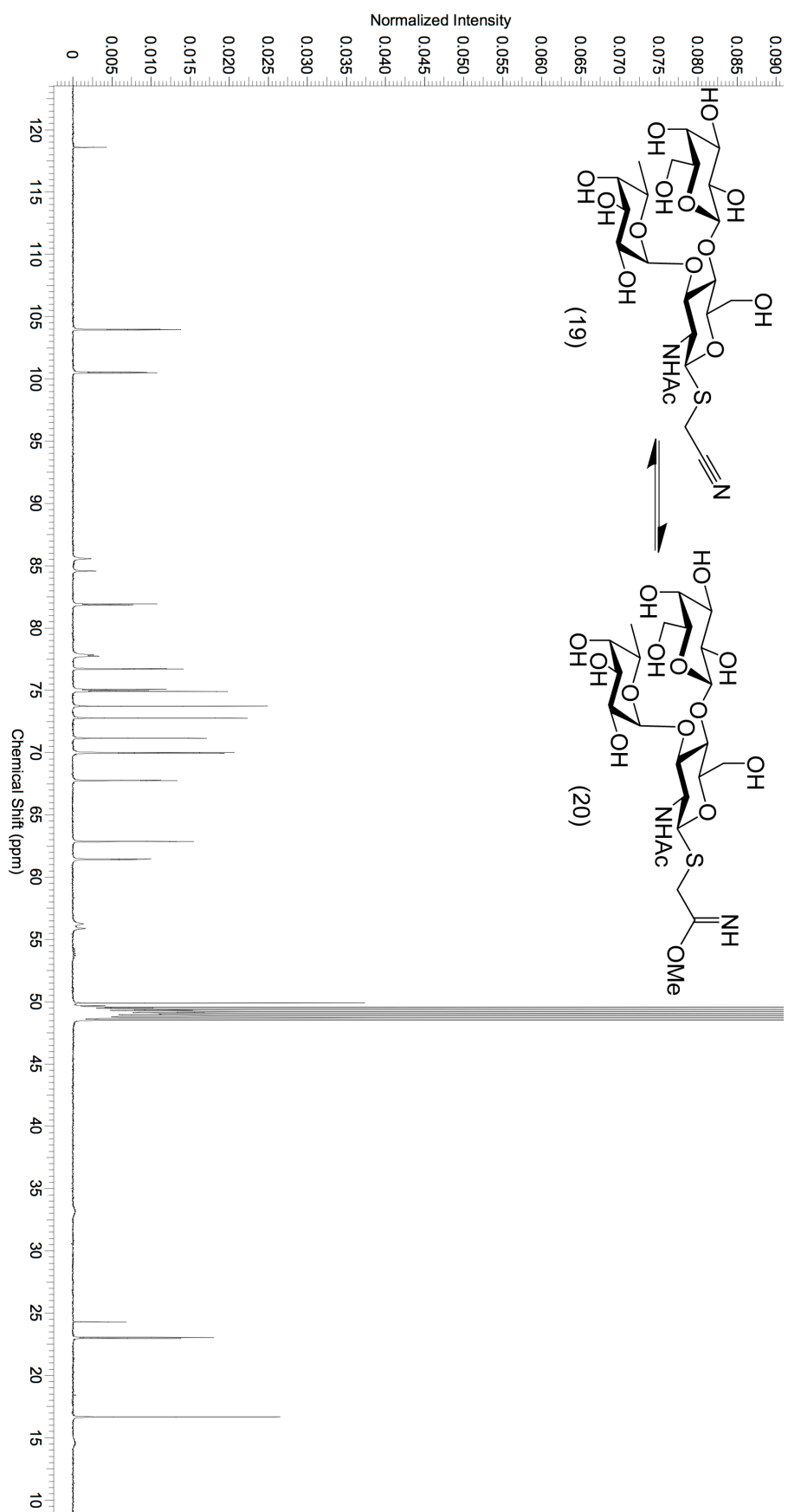












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3. Adenovirus modification with IME sugars

3.1 Introduction

3.1.1 Adenovirus

Adenovirus (Ad) was first identified in the 1950's from adenoid tissue-derived cell cultures¹. Since then, 57 serotypes of Ad have been recognised in humans which are capable of infecting multiple organ systems². Although Ad infection is mostly asymptomatic, Ad infection in immune-compromised patients can cause life-threatening, multi-organ disease³. The Ad serotype most commonly employed as a DNA vaccine vector is species C Ad, also called HAdV-C5 (Ad5)⁴⁻⁸. The genome can be divided into early (E1-E4), which express before viral DNA replication, and late (L1-L5) transcripts. In order to increase the safety of Ad as a therapeutic tool, regions of the early genome have been removed, rendering the Ad replication incompetent. The first generation of Ad vectors were E1 deleted, however when therapeutic DNA was inserted the genome was too large and the Ad became unstable. Later Ad vectors were both E1 and E3 deleted, permitting the inclusion of up to 8Kbs of foreign DNA, whilst retaining Ad stability^{9,10}.

3.1.2 Adenovirus structure and function

The icosahedral structure of Ad, with fiber proteins extending from each of the 12 vertices, was first described in the early 1960s^{11,12}. The 11 structural proteins of Ad were first isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were named in order of increasing molecular mass¹³(**Table 1**). The entire structure of Ad was first pieced together as a whole by Stewart *et al* in 1993¹⁴ (**Figure 1**).

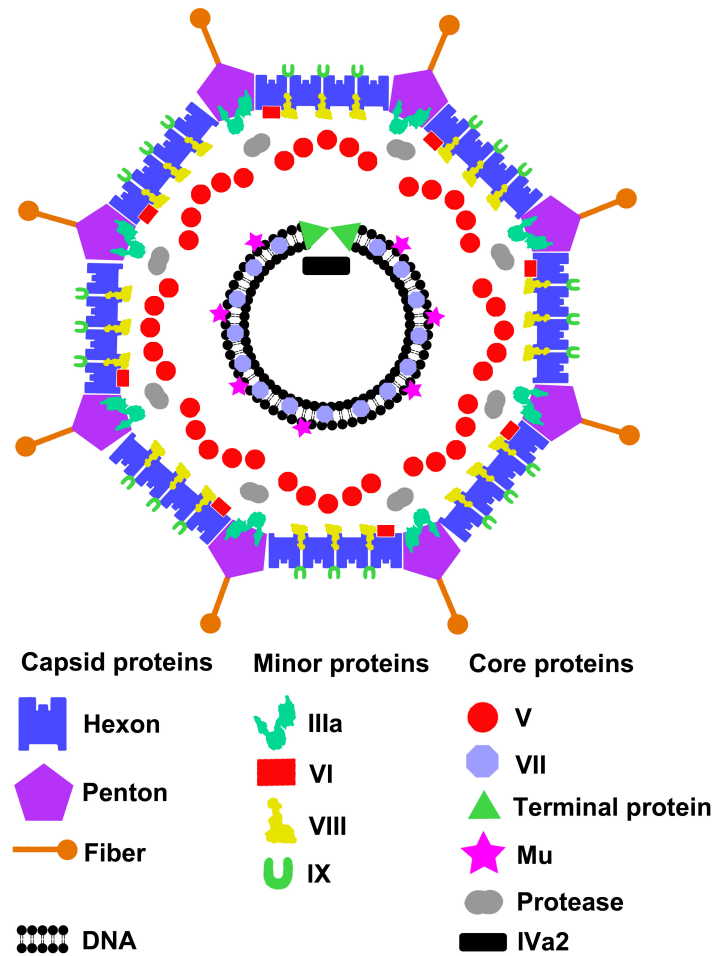


Figure 1 - Illustration of the structure of Ad based on cryo-electron microscopy and crystallography. The minor proteins are associated with the capsid and the core proteins with the viral DNA. Adapted from the structure proposed by Russell *et al*¹⁵.

Table 1 – Adenovirus structural proteins. Adapted from Rux *et al* and Stewart and Burnett *et al*.

Polypeptide	Molecular mass of monomer (Da)	Number of residues in monomer	Biochemical copy number of monomer	Copy number of protein in current model
II (hexon)	109,077	967	720 +/- 7	240 Trimers
III (penton base)	63,296	571	56+/-1	12 Pentamers
IIIa				
External portion	41,689	376		
Internal portion	21,846	194		
Total	63,535	570	68 +/- 2	60 Monomers
IV (fiber)				
N terminus 2/8 of shaft	13,912	133		

6/8 of shaft C terminus	48,048	449		
Total	61,960	582	35+/- 1	12 Trimers
V (core)	41,631	368	157 +/- 1	-
Terminal (core)	~55,000	~500	2	
VI				
Ordered portion	9,773	91		
Disordered protein	12,345	115		
Total	22,118	206	342+/-4	60 Hexamers
VII (core)	19,412	174	833+/-19	
VIII	15,390	140	127+/-3	
IX	14,339	139	247+/- 2	80 Trimers
u ^f (core)	~4,000	~36	~104	

Ad is assembled from 252 subunits; 12 pentamers and 240 hexamers¹⁶. In 2010 the atomic structure of whole Ad particles was described by both crystal X ray crystallography (at 3.5Å resolution) and cryo-EM, revealing interactions between the capsid proteins which had been previously been studied in isolation^{17,18}.

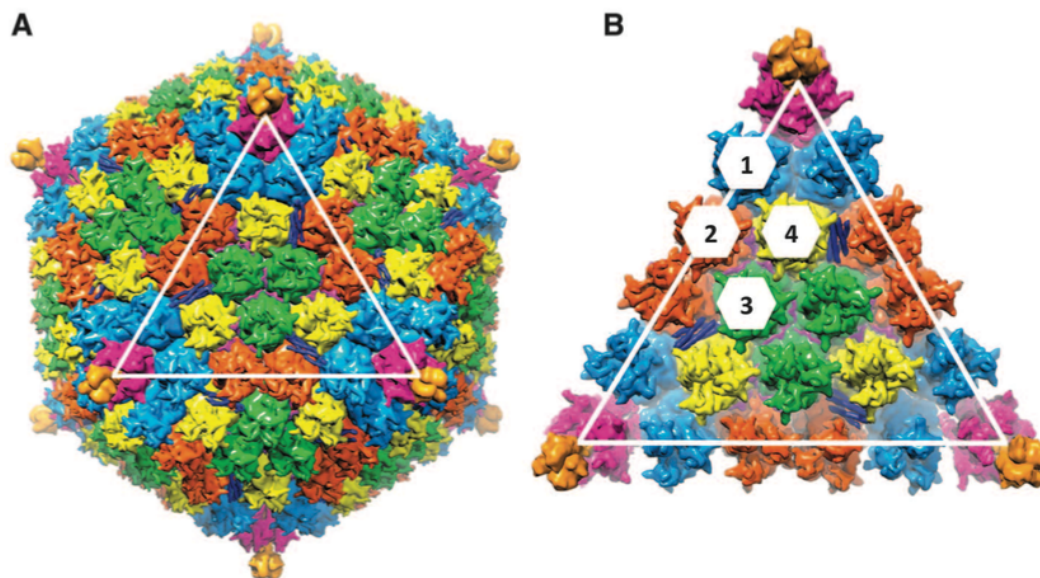


Figure 2 – a) Surface rendering and subunit associations of Ad. The icosahedral asymmetric unit is composed a pentamer subunit and four hexon trimers (hexamer) ‘Cement’ proteins on the outside of the capsid are shown in dark blue (four-helix bundle) and magenta (IX triskelion). b) One face of the icosahedron marking the four different symmetrical states of the hexamer. Adapted from Reddy *et al*¹⁹.

The hexamer is composed of a trimer of hexon proteins (protein II) that form an oligomer, with pseudo 6-fold symmetry at its base, and a trimeric structure that protrudes from the surface of the capsid¹⁸. The hexon protein accounts for 63% of the total protein mass of the Ad viron²⁰. Sequence analysis has shown that hexon loops contain hypervariable regions which vary between Ad subtypes²¹. These regions have therefore been the most targeted during mutation studies^{22,23}; chimeric Ads have been also created by replacing Ad5 hexon proteins with those from other Ad serotypes^{23,24}.

The penton protein (protein III) is found at each of the 12 vertices of the Ad capsid where it forms a pentamer complex, called the penton base. The fiber, formed of a trimer of protein IV, protrudes from the centre of the penton base. The penton and fiber are responsible for Ad cell surface attachment and subsequent internalisation. The protruding fiber proteins anchor the virus to the cell surface via interaction with CAR^{25,26}. The structure of the fiber can be described as three domains: a tail domain fixes the fiber into the penton base, followed by a shaft of repeating units, which is finally capped by a C-terminal, globular knob domain²⁷. The knob domain is responsible for the interaction with CAR^{25,26}. The shaft of the fiber contains hinge regions, resulting from the insertion of extra residues in the beta-turns of the repeating units. These hinges allow the fiber sufficient flexibility for the secondary binding event between the penton base and the cell surface²⁸. Ad also contains a number of minor coat proteins that are thought to associate with the internal surface of the Ad capsid²⁹. This group of proteins includes protein IIIa (which co-elutes with the penton protein on SDS-PAGE), and is positioned below the penton base within the capsid³⁰.

As the virus anchors onto the cell surface through the CAR-fiber interaction, the penton base binds to α_v integrins, allowing the virus to internalise via endocytosis³¹. This interaction is dependent on the highly conserved Arg-Gly-Asp (RGD) sequence, located on the exterior of the penton base. It is thought that the multiple RGD peptides displayed on the penton base cause integrins to cluster on the cell surface and therefore induce the endocytosis of Ad^{21,32-35}. Ad is internalised into endosomes via

clathrin-coated pits, the virus partially disassembles and escapes into the cytoplasm. Ad is then transported, via microtubules, to the nuclear pore complex, releasing its genome into the nucleus³⁶⁻³⁹.

Reports have demonstrated that lysine residues are either involved in or are in close proximity to all of the described virus-cell interaction sites⁴⁰⁻⁴³. Ad5 has minimal natural glycosylation, with a single O-GlcNAc at Ser-109 of the fiber^{44,45}, which has no reported functional role. Hence, Ad5 is a blank canvas for the chemical installation of targeting carbohydrates. We therefore reasoned that chemical alteration of the adenovirus capsid proteins with carbohydrates might allow direct modulation of entry: controlling (even curtailing) normal pathways and enabling novel lectin binding.

3.1.3 Prior attempts to artificially glycosylate Ad.

Non-targeting polysaccharides have been grafted onto the Ad capsid surface⁴⁶. Espenlaub *et al* coated Ad with poly-mannose via reductive amination, resulting in multiple covalent bonds to Ad residues. One advantage of this method is that no linking groups are required- non-synthetic carbohydrate polymers from natural sources can be conjugated directly. However, the purity of these products can be limiting; many are heterogeneous mixtures of different lengths and branching isoforms. The Carrico group have used metabolic incorporation of an unnatural azido sugar at the single glycosylation site of the Ad fiber, allowing subsequent alkyne-azide cycloaddition to attach targeting ligands. However, the group did not target lectins using this method^{47,48}. The chemical glycosylation of Ad with imidate sugars was first reported by the Davis group in 2005⁴⁹. This early work demonstrated the durability of the technique using mannose and galactose imidate sugars. Pearce *et al* also conducted some cellular retargeting studies and demonstrated the reduction of transfection in non-target cell lines⁴⁹. This project expands on this initial work and will explore more complex targeting saccharides and analyse the altered

tropisms of these new 'glyco-Ads' in greater detail. By coating the Ads with LeX or mannose the glyco-viruses was targeted to DCs via DC-SIGN. This sugar coating abrogates Ad's natural CAR(+) dependent infection, thus limiting tropism to DCs. The ability of the glyco-Ads to evade neutralisation by anti-Ad immune responses was also investigated. Together, glyco-Ad DC targeting and immune evasion improves the effectiveness of Ad as a DNA vaccine vector.

3.2 Results

3.2.1 Adenovirus stability

The IME LeX reagent is activated by reaction with catalytic amounts of NaOMe, which is not neutralised before the reagent is dried (**Chapter 2**). The addition of this reagent will therefore expose the Ad to an increase in solution pH. To assess the stability of Ad to various pH values, Ad expressing GFP (*gfp*-Ad) was dialyzed into a buffer mixture of MES, HEPES, TAPS, and CAPS at increasing pH values. The Ads were then exchanged into storage buffer (PBS, 50mM HEPES, 10% glycerol) and were analysed for denaturation by nanotracker and infectivity assay. The benefit of nanotracker over other size distribution measurements, such as dynamic light scattering (DLS), is the ability to assess the relative concentration of the size species. The size distribution of Ad at pH 7 was relatively monodisperse, with an average particle size of 105 nm, in accordance with the literature value⁵⁰. However, at lower pH and in solutions greater than 8.5 these particles are not present, suggesting that the virus is no longer intact (**Figure 3**). This result was confirmed when the pH-exposed *gfp*-Ads were used to infect Raji cells (a lymphocyte cell line). The virus infectivity was assessed by the number of cells expressing GFP, as measured by flow cytometry. Based on this assay, the *gfp*-Ad infectivity remains stable between pH values of 7-9 (**Figure 4**).

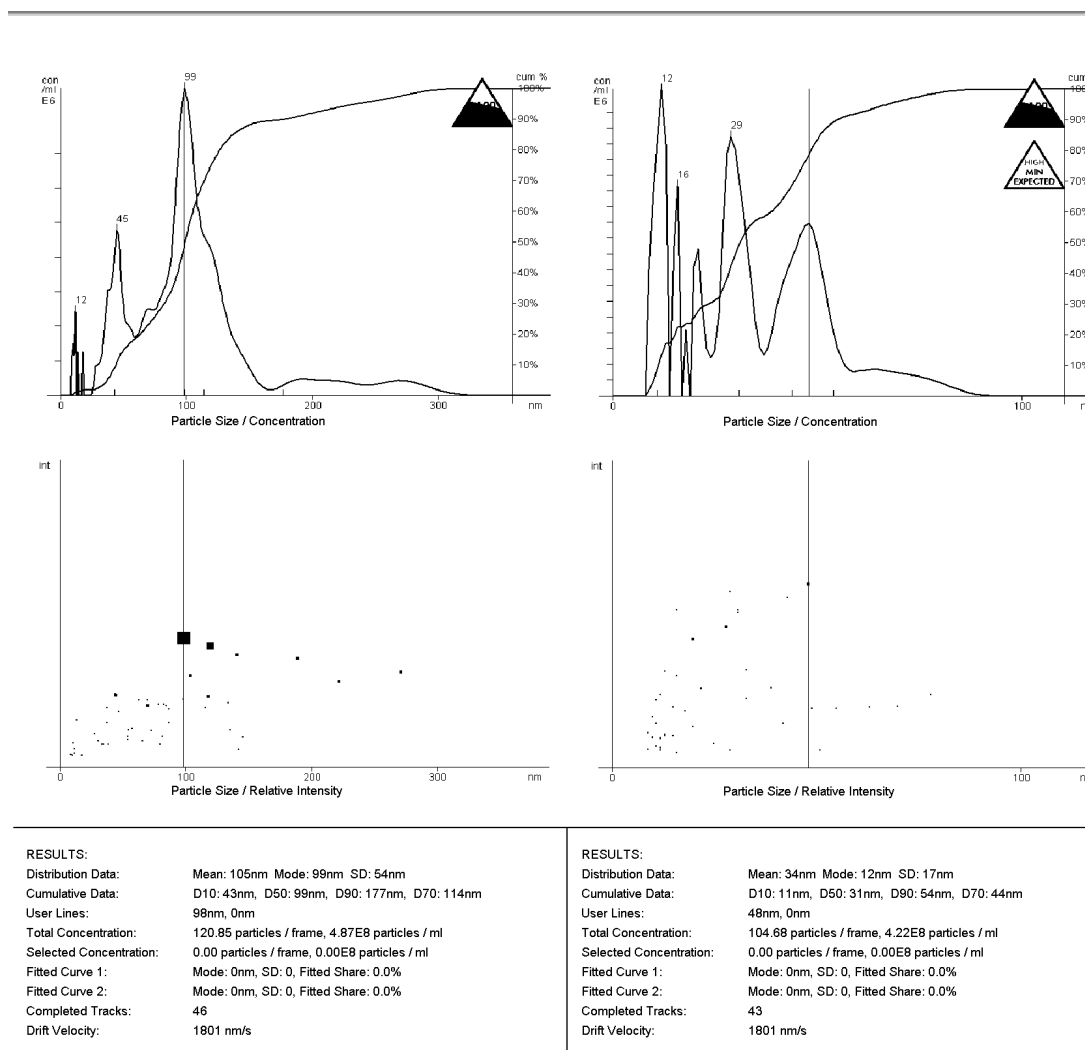


Figure 3 - Examples of nanotracker results from Ad samples, exposed to buffers at pH 7 (left) and 10 (right). The extent of any virus denaturation was measured by nanotracker, which uses the rules of Brownian motion to assess particle size. The Ad sample at pH 7 contains particles at 105nm, consistent with the literature value for Ad⁵⁰. The sample at pH 10 however, does not contain any monodisperse particles.

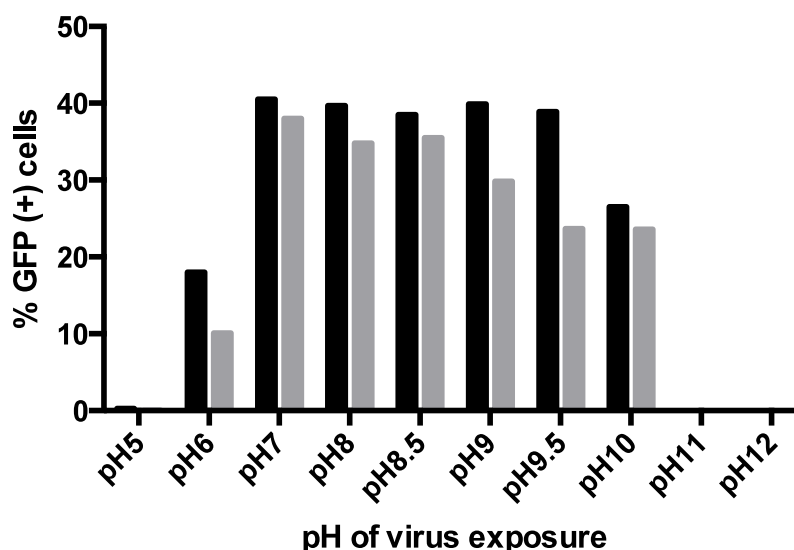
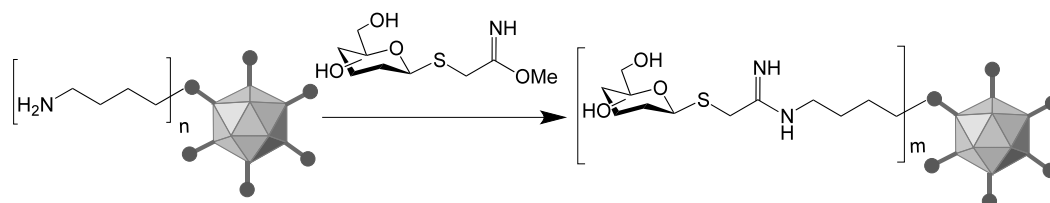


Figure 4 - Raji cells were infected with *gfp*-Ad samples that had been exposed to buffers of increasing pH values. The number of GFP expressing cells were measured 24 hours post infection and the experiment was performed in biological duplicate. Ad remains infective between pH 7-9.

3.2.2 Characterization of modified Adenoviruses



Ad samples were exchanged into 0.6M TAPS buffer at pH 7 before the addition of the sugar IME reagents (increasing the pH value to 9) at various equivalents/lysine ($n=22,000/\text{Ad}^{51}$). The samples were then left to react for 2 hours at room temperature, before the resulting glyco-Ads were transferred into storage buffer by dialysis. The concentration of the viruses (virus particles (vp)/mL) was then calculated based on the concentration of DNA from denatured glyco-Ad samples. In brief, the samples were exposed to sodium dodecyl sulfate (SDS) and heated, before the addition of Picogreen® reagent, which fluoresces when bound to DNA. The DNA concentration was then extrapolated from a standard curve and divided by the known molarity of DNA from a single virus particle (**Figure 5**)⁵².

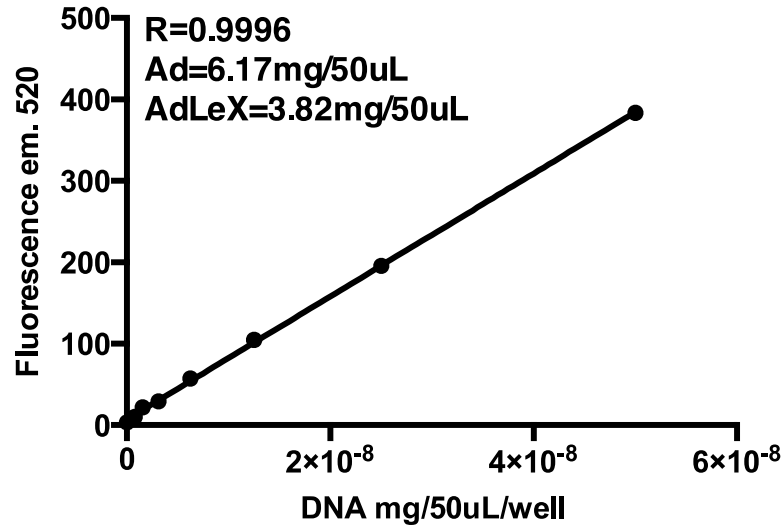


Figure 5 - Fluorescence (ex.485/em.520) of DNA standard curve after the addition of Picogreen® reagent. The concentration of DNA from denatured virus samples was then extrapolated.

The glyco-Ads were then analysed by SDS-PAGE to measure the extent of protein mass shift due to sugar modification. The gels were visualized with silver stain and mass increases were measured by densitometry (**Figure 6 and 7**). The IME LeX modified the external capsid proteins; hexon, penton, protein and the fiber, but not protein IIIa. The smaller monosaccharide mannose and galactose IME also modified some of the internal proteins, in particular protein V.

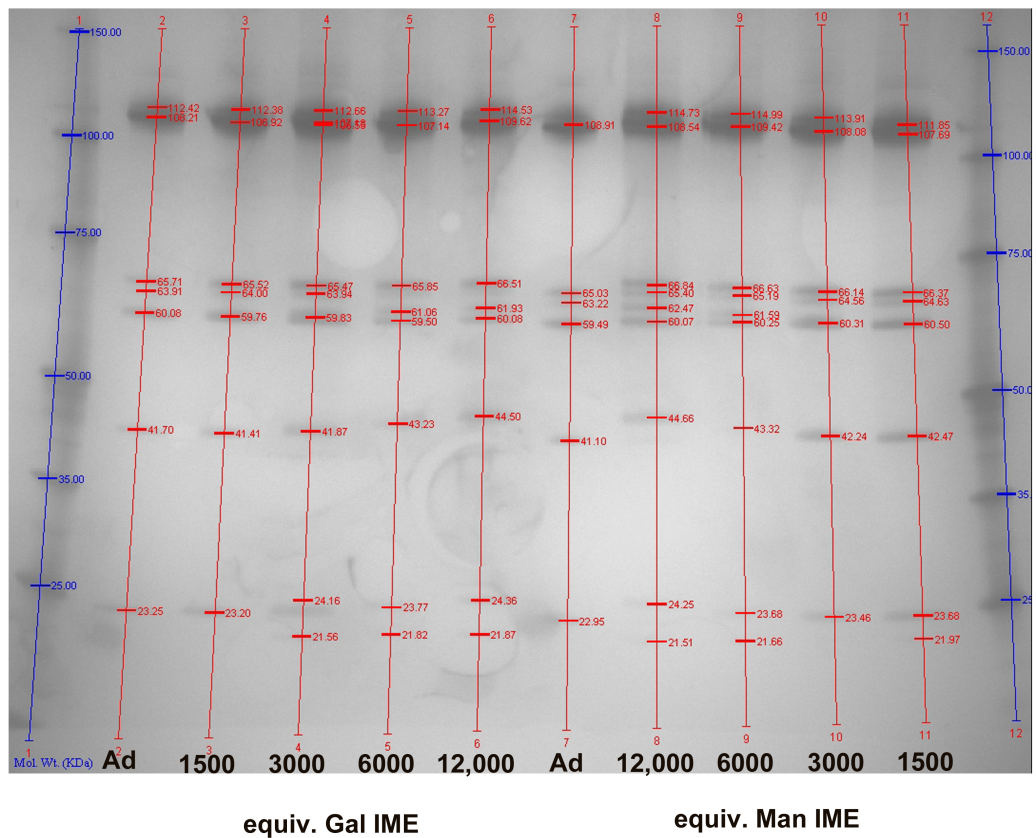
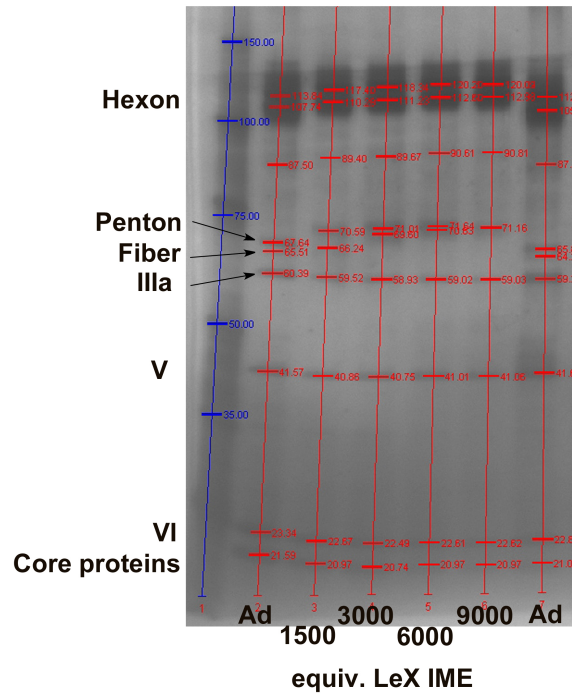
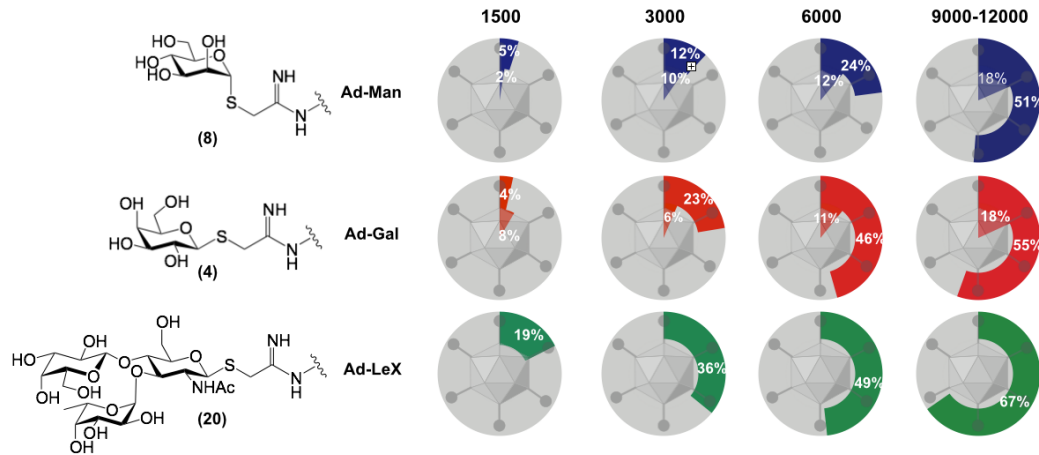


Figure 6 - SDS-PAGE of Ads modified with increasing equivalents of LeX, galactose or mannan IME reagents. The gels were visualized with silver stain and the molecular weight of the bands measured against protein standards by densitometry.

Equivalents of IME sugar/lysine (22,000/virus)



Mannose IME

Equiv/lysine Protein	1500		3000		6000		12000	
	No. Sugar	x copy No.	No. Sugar	x copy No.	No. Sugar	x copy No.	No. Sugar	x copy No.
Hexon upper	1.1	803.2	3.2	2323.5	5.7	4073.3	10.7	9810.4
Hexon lower	0.0	-	0.0	-	0.0	-	2.9	-
III penton	2.0	109.3	1.8	98.2	3.3	182.9	5.9	330.2
IIIa	3.1	108.8	2.9	100.4	10.5	366.7	13.1	458.8
Fiber	1.1	73.1	1.4	92.1	6.3	425.3	9.7	661.0
V	1.2	193.9	3.1	481.6	8.5	1332.3	13.5	2126.7
VI	1.0	340.6	4.8	1648.7	3.3	1117.3	5.6	1921.2
Capsid total	1094.4 (5.0%)		2614.2 (11.9%)		5048.3 (22.9%)		11260.4 (51.2%)	
Core total	534.5 (2.4%)		2130.3 (9.7%)		2449.1 (11.9%)		4047.9 (18.4%)	

Galatose IME

Equiv/lysine Protein	1500		3000		6000		12000	
	No. Sugar	x copy No.	No. Sugar	x copy No.	No. Sugar	x copy No.	No. Sugar	x copy No.
Hexon	0.0	0.0	5.9	4274.1	10.2	8835.1	11.5	10699.6
Hexon lower	0.0	-	0.0	-	2.0	-	3.4	-
III penton	5.3	299.0	4.4	247.6	6.4	357.0	7.2	403.8
IIIa	5.6	196.6	5.3	186.9	7.8	274.7	8.7	304.0
Fiber	4.0	273.6	3.3	222.2	8.4	598.9	11.9	807.2
V	5.5	856.9	4.5	713.1	8.8	1388.6	14.2	2226.8
VI	2.9	994.7	2.0	694.9	2.9	994.7	5.2	1771.3
Capsid total	769.2 (3.5%)		4930.8 (22.4%)		10035.7 (45.6%)		12214.7 (55.5%)	
Core total	1852.6 (8.4%)		1408.0 (6.4%)		2383.3 (10.8%)		3998.1 (18.2%)	

Lewis-X IME

Equiv/lysine Protein	1500		3000		6000		9000	
	No. Sugar	x copy No.	No. Sugar	x copy No.	No. Sugar	x copy No.	No. Sugar	x copy No.
Hexon	0.0	2976.0	6.3	7572.0	8.2	10068.0	10.9	13704.0

<i>Hexon lower</i>	4.1	-	4.3	-	5.8	-	8.1	-
<i>III penton</i>	9.7	545.1	4.9	275.3	5.6	314.5	6.7	373.3
<i>IIIa</i>	9.7	340.7	1.2	42.6	6.8	238.6	8.5	298.7
<i>Fiber</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>V</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>VI</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>Capsid total</i>	4183.9 (17.6%)		7930.1 (35.9%)		10846.1 (48.3%)		14657.6 (65.3 %)	
<i>Core total</i>	-		-		-		-	

Figure 7 –Ad capsids were successfully modified with lysine-reactive IME sugars. The structures introduced are shown on the left hand side of the panel. The concentric pie charts represent the percentage coverage on both external and internal lysine residues. The smaller monosaccharides Man and Gal were observed to modify internal Ad proteins. The percentage of sugar coverage based on the number of sugar modifications (m) (estimated by SDS-PAGE band shift) over the total number of lysine residues in all viral proteins (n=22,000). Increasing equivalents of IME sugar/lysine were employed.

The size (diameter) increase of the Ad virus particles after IME reaction was assessed by DLS; the viral concentrations were too low to allow use of the nanotracker method. The diameter of glyco-Ads was found to increase while remaining monodisperse (**Figure 8**).

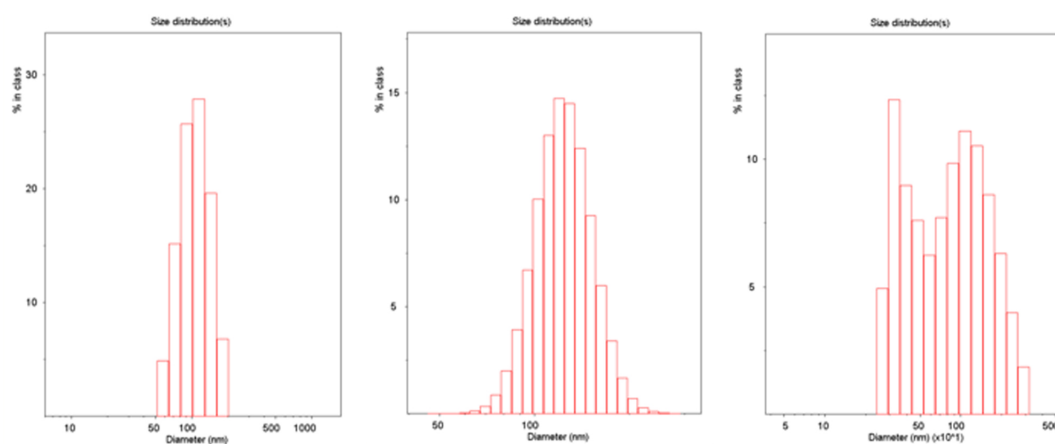


Figure 8 - DLS measurements of Ad, Ad-LeX (3000 equiv) and a sample of denatured Ad, diluted in PBS.

Native agarose gel presents a viable alternative to the denaturing SDS PAGE as the Ad virus has been shown to remain stable in the matrix¹⁰. A 1% agarose gel was able to resolve between the modified and unmodified,

intact viruses - the Ad-LeX band migrated to a lesser extent (**Figure 9**). A denatured Ad sample (as previously assessed by nanotracker/DLS) was also included and found to give a smear rather than a defined band.

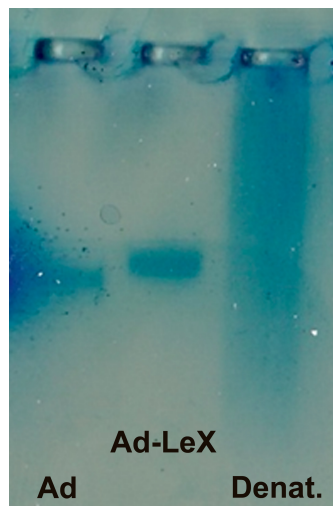


Figure 9 - A 1% 0.5cm agarose gel loaded with samples of Ad, Ad-LeX (3000 equiv) or denatured Ad, in of DNA loading buffer.

3.3 Discussion

Ads were successfully modified with mannose, galactose and IME LeX sugars, with up to 73% of total lysine residues (22,000/vp) glycosylated. The smaller monosaccharides modified both external and internal Ad capsid proteins, whereas the LeX tri-saccharide only reacted with the exterior capsid proteins. As the virus has been shown to remain intact after the IME reaction, the Ad capsid must be somewhat permeable. The extent of viral protein glycosylation was assessed by SDS-PAGE mass gel shift; this method allows the use of a small amount of the modified virus. However, measuring mass increase by protein band shift is not particularly precise, with an estimated error of $\pm 2000\text{Da}$. Ideally, the glyco-viruses would be analysed by mass spectrometry, both whole protein mass followed by digestion and tandem mass spectrometry. This would allow the precise measurement of the number of sugars per capsid protein and their locations. Such detailed information on the patterns of the IME

glycosylations of Ad might also contribute to understanding subsequent biological results.

3.4 Experimental

General methods. Picogreen assay® analysis was determined using a Wallac Victor 1420 plate reader. DLS analysis was performed using a Malvern Zetasizer 3000HS. Biological material was handled in BIO2+ lamina flow hoods. Electrophoresis was run using BioRad gel units and staining was performed with SilverXpress® silver staining kit (Life Technologies). Raji cells (NIH AIDS program) were maintained in complete RPMI medium (heat inactivated 10% fetal calf serum, 2 mM L-glutamine). HEK293 cells were maintained in complete DMEM (high glucose, 10% FCS). Flow cytometry analysis was conducted on a FACSCaliber™ by BD Biosciences.

Virus production. HEK293 cells expressing adenovirus early E1 protein were cultured in complete DMEM. Once the cells were confluent they were counted and exchanged into HyperFlasks, these were left at 37°C, 5% CO₂ for 3 days. The cells were infected with E1/E3 deleted Ad at 100MOI and again left at 37°C for 3 days, after which the cells showed loss of confluency but were not lysed. The cells were then centrifuged (4750rpm, 5 min, 4°C) and the flasks were washed with PBS, which was also centrifuged to obtain cell pellets. These were combined and diluted to 20mL (enough for 4 columns) with media containing 2% FBS. This solution was then subjected to three freeze-thaw cycles, by placing the flask into liquid N₂ and then a water bath at 37°C. After the final thaw, 200µL of *n*-butanol was added and left for 45 min at 4°C. The flask was centrifuged (2000xg, 5 minutes), separating the lysed cells from the media containing the virus. An ultracentrifuge tube was loaded with 3mL of 1.32 Ω CsCl (10mM Tris pH 8), underplayed with 2mL 1.45 Ω CsCl (10mM Tris pH 8). An additional top layer of 2mL 40% aqueous glycerol was added and 5mL of the supernatant

was carefully layered on top. The tubes were centrifuged at 25,000rpm with slow deceleration for 1 hour 30 min at 4°C. The virus band (**Figure E1**) was removed from the tube using a needle and syringe; in total about 10mL of the virus solution was removed and diluted up to 20mL with PBS. This was loaded into a 100KDa MWCO dialysis cassette and left in PBS for 15 mins, the PBS was exchanged and the cassette left overnight at 4°C. The virus was removed and 1µL Benconase Nuclease was added (Novagen stock concentration 25 units/µL), the enzyme was left for 1 hour at room temperature. The virus solution was then put on another identical CsCl column and the virus band was removed and loaded into a dialysis cassette and left in PBS (20 min, 4°C with two exchanges). To the final dialysis 50mM HEPES buffer was added (20 min 4°C). Finally 10% sterile glycerol was added to the virus samples. The virus was stored at -80°C. To calculate the virus concentration the UV absorbance at 280 and 260nm was measured in accordance with a protocol developed by Maizel *et al* ⁵³. To denature the virus, samples with an expected concentration of 0.5-2 x 10¹² particles/mL, were combined with equal volumes of 0.2% SDS buffer (for concentrations expected to be >2 x 10¹² particles/mL samples were diluted to approximately 1x10¹² particles/mL). Absorbance was then read at 320, 280 and 260nm. To calculate the corrected Ad₂₆₀/A₂₈₀ ratio: A₂₆₀ corrected = A₂₆₀ - A₃₂₀ and A₂₈₀ corrected = A₂₈₀ - A₃₂₀, corrected ratio = corrected A₂₆₀/A₂₈₀ corrected. (A₃₂₀= the absorbance used for background and scatter correction). Calculate the particle count according to Maizel *et al* in particles/mL as follows:

$$\text{Virus concentration [virus particle/mL]} = 1.1 \times 10^{12} \times \text{corrected } A_{260} \times \text{dilution factor}$$

Where: 1.1×10¹² = the number of virus particles per mL per 1 AU at 260 nm. The acceptance criteria for a measurement are as follows i) none of the absorbance readings should be > 0.25 AU, ii) the difference between A₂₆₀ and A₃₂₀ should be < 0.05 AU, iii) the absorbance reading at 260 nm should be between 0.1 and 1.0 AU, iv) the absorbance reading at 320 nm should be

< 0.05 (if > 0.05 , interference from light scattering will be too high, v) the average ratio of 260/280 should be between 1.2 – 1.4. The UV method of Ad concentration measurement is therefore only reliable at high concentrations of Ad. After modification Ad is too dilute and concentrations are measured using a Picogreen fluorescence assay.

Virus band



Figure E1- A CsCl column of adenovirus separated from lysed cells after centrifugation at 25,000 rpm for 1 hour 30 minutes.

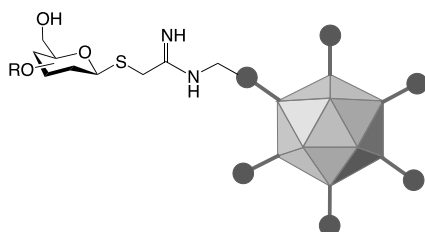
Virus pH exposure. *gfp*-Ad 20 μ L (1.02 vp/mL) was dialyzed into a buffer mixture of MES, HEPES, TAPS, and CAPS at 0.1M each at pH 5, 6, 7, 8, 8.5, 9, 9.5, 10, 11, and 12 for 4 hours at RT. The virus dialysis cassettes were then transferred into sterile PBS 50mM HEPES and left to exchange back into virus storage buffer at 4°C overnight. For all samples, with the exception of the samples to be used for nanotracker analysis, 10% glycerol was then added to the samples and the virus samples stored on ice.

Nanotracker analysis. Virus samples were injected directly into the NanoSight unit with a 1mL sterile syringe (approximately 300 μ L filled the unit). The NanoSight recorded 60-second sample videos, which were then analysed with Nanoparticle Tracking Analysis Analytical software.

Raji infection. 5×10^4 cells/well/200 μ L were plated in 'V'-bottomed 96 well plates in RPMI 2% FCS. 10 μ L of virus dilutions (1000MOI), in 2% media, were then added and left for 2 hours at 37°C. The cells were then centrifuged (1200rpm 5min 4°C) and the media was removed by aspiration to be replaced with 2% FBS media. This step was repeated a further two times. The cells were left for 2 days at 37°C before being analysed for GFP expression by flow cytometry.

Native gel. A 50mL solution of 1% w/v agarose powder in Tris-Acetic acid EDTA buffer (TAE 10X concentrate BioRad) was heated in a microwave (700 Watt, 1 minute). This was then poured into a cast and set for 40 minutes to yield a gel of 0.5 cm depth. Samples were loaded with DNA loading dye, which contains two types of dye and glycerol, but no SDS. The gel was then run in TAE buffer for 60 minutes at 60 volts and left to stain in Coomassie Blue stain overnight.

General procedure for adenovirus modification



All adenovirus (Ads) used were E1/E3 deleted, replication-incompetent viruses. Viruses were exchanged into 0.6M TAPS pH 7 by dialysis at 4°C. Reagent (19/20, 7/8 or 3/4) was dissolved in 0.6M TAPS buffer (pH 9) and the resultant solution immediately added to the virus. Gentle pipetting was used to mix the solution. The sample was shaken and left at room temperature for 2 hours. The samples were purified by exhaustive dialysis against PBS, 50mM HEPES pH 7.5, at 4°C. To the final volumes of virus sample 10% glycerol was added and the viruses stored on ice (short term max. 3 weeks) or at -80°C.

Viral particle concentration. Virus samples were diluted (10µL into 90µL) with sterile PBS + 0.05% SDS and heated at 56°C for 1 hour. 50µL of lysed virus samples were then combined with 50µL Picogreen reagent in a black-sided 96 well plate (Quanti-iT Picogreen assay®, Life technologies). Fluorescence intensity was measured immediately ex. 485nm em. 515nm. Final DNA concentrations were extrapolated from a DNA standard curve, had prepared in parallel to the virus samples. The viral DNA concentration was then divided by the mass of one adenovirus genome (2.3×10^6 g/mol⁵²) resulting in a viral titer in virus particles (vp) /mL).

Dynamic light scattering (DLS) analysis. The unmodified and modified viruses were analyzed by dynamic light scattering. First, the cuvettes were cleaned with air dust. Then, virus samples (4.0 µL) were added into sterile and filtered PBS buffer (600 µL, pH 7.4). The samples were then analyzed with the DLS instrument (polydispersity index and average diameter) using a laser wavelength of 532 nm at a temperature of 25°C. Refractory index and viscosity of water at 25°C were used.

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4. *In vitro* studies of glyco-Ads as DNA vaccine vectors

4.1 Introduction

4.1.1 Ad tropism and bio-distribution

The two-step internalisation route of Ad involves the initial anchoring of the virus to the cell surface via the fiber-CAR interaction, followed by binding between the penton base and α_v integrins¹. In one study 78% of human platelets stained positive for CAR, and in mice blood platelets interact with Ad, activating platelet leukocyte aggregate formation². This interaction results in virus sequestration to the reticulo-endothelial system of the liver³.

However, CAR expression alone does not seem to dictate Ad tropism *in vivo*; other less well-defined interactions are thought to be equally important for infection. Heparan sulphate glycosaminoglycans (HSGAGs) can mediate CAR-independent infection by Ad5; mediated by a Lys-Lys-Thr-Lys motif on the fiber shaft⁴⁻⁸. In the clinic, excessive liver tropism limits the maximum dosage of Ad vectors. Reports have demonstrated that ablation of the HSGAGs receptors on the fiber can prevent liver infection in mice⁹.

Ad has also been reported to interact with factors in blood that alter the bio-distribution and transduction of the virus¹⁰. Kinetic studies demonstrated that almost all Ad5 was cleared from the mouse bloodstream within 3 minutes of I.V administration¹¹. After 60 minutes of incubation of Ad5 in whole human blood *in vitro*, more than 98% of the virus particles were attached to the surface of erythrocytes¹². Lyons *et al* also observed that in *ex-vivo* human blood from individuals injected with Ad5, over 90% of Ad5 was found to be stably associated with the erythrocytes¹³. They concluded that the Ad-erythrocyte interaction directly decreased the ability

of the virus to access to extra-vascular target cells, thus limiting the overall transgene expression.

Neutrophils present in the blood have also been shown to bind and internalise Ad¹⁴. Upon intravenous (I.V.) injection with Ad5, neutrophils are rapidly recruited to the liver, 25% of which were found to have interacted with the virus. Muruve *et al* report that this massive influx of Ad-bearing neutrophils is responsible for some of the Ad-induced hepatic injury seen after I.V. injection¹⁵. The Ad-neutrophil interaction was dependent on the presence of complement and antibodies, suggesting the need of a facilitating protein bridge between Ad and the neutrophil¹⁴.

The blood components coagulation factors FVII, FIX and FX have been reported to increase hepatocyte transduction by Ad5¹⁶⁻¹⁸. Mice pre-dosed with warfarin did not show liver transduction after I.V injection of Ad. The administration of FX to these animals restored Ad liver transduction¹⁹. FX binds to Ad via the hexon, the most abundant Ad capsid protein^{20,21}. The receptor responsible for the coagulation factor dependent hepatocyte uptake was confirmed as the HSGAGs²². Ad has also been shown to have a direct interaction with HSGAGs, binding via the fiber in the absence of blood factors^{23,24}. Complement factor 3 and 4 also interact with the Ad capsid, independently from antibodies, and promote liver transduction²⁵⁻²⁷. Complement dependent enhancement of Ad liver transduction also results in an increased inflammatory response, which can result in toxicity²⁸.

Kupffer cells (KCs), the liver resident macrophages, are the first line of defence against systemic circulating viruses due to their localization within the liver sinusoids. KCs therefore have a strong influence on Ad bio-distribution and are thought to be responsible for a large proportion of viral clearance *in vivo*²⁹⁻³¹. I.V. administration of Ad can be toxic and potentially lethal - it is thought that this reaction could be due to the uptake of Ad by KCs. This uptake can be direct, through the scavenger receptor A (SR-A) or indirect, via the uptake of Ad-platelet aggregates and Ad-IgM-complement complexes^{3,32,33}. Upon Ad internalisation, KCs are strongly activated,

releasing IL-6 and TNF- α , followed by necrosis³⁴. Recent work by Piccolo *et al* also indicated that sinusoidal endothelial cells have a role in the mechanisms of Ad associated toxicity³⁵.

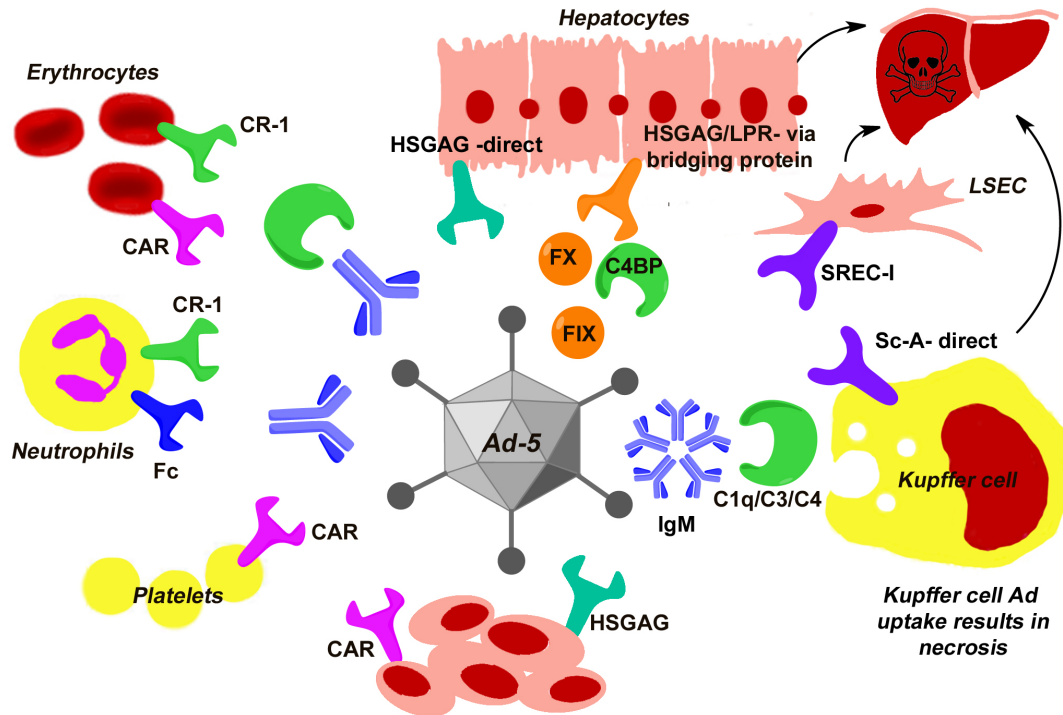


Figure 1 - Neutralisation and sequestration mechanisms of Ad *in vivo*. Neutrophils bind adenovirus directly through CR-1 and indirectly by Fc-IgG-Ad interactions. Erythrocytes also express CR-1, as well as the primary Ad receptor, CAR. Ad can also be bound by heparin sulphate glycosaminoglycans (HSGAGs) expressed by many cell types. Co-factors FX, FIX and complement C4BP can bind to Ad and act as bridging ligands, enhancing hepatocyte infection. Kupffer cells in the liver readily sequester Ad directly by scavenger receptors (Sc-A) and indirectly through IgM/complement and platelet complexes. Kupffer cell Ad uptake results in necrosis. A third type of liver cell, sinusoidal endothelial cells (LSEC), also internalise Ad through the SREC-I scavenger receptor. Together, these liver interactions with Ad can result in toxicity^{3,4,16-18,22,26-34}.

Structural analysis and mutation experiments suggest that lysine residues on the capsid proteins are either involved in or are in close proximity to these Ad-protein and Ad-cell interaction sites^{20,36-40}. The IME-modified glyco-Ads developed here could therefore prevent the undesirable cell infections described previously. In order to investigate the

retargeting of the glyco-Ads the cell line A549, which expresses CAR, will be used as a naturally adenovirus-susceptible cell. Ideally, the chemically introduced sugars will interrupt the CAR-Ad interaction and abrogate the infection of these non-target cells. Concurrently, the sugar might enable the Ad to bind to the reciprocal cell surface lectin, allowing infection of previously refractory cells.

4.2 Results

4.2.1. Retargeting from CAR(+) A549

Glyco-Ads expressing GFP transgene and modified with increasing equivalents of mannose (*gfp*-Ad-Man), galactose (*gfp*-Ad-Gal) or LeX (*gfp*-Ad-LeX) IME sugars were used to infect A549 lung epithelial cells. 24 hours later the levels of infection were measured by the percentage of A549 cells expressing GFP. The infection of the cells was conducted at 100 multiplicity of infection (MOI i.e. 100 virus particles per cell). Apoptosis of cells was not detected after infection with any of the glyco-Ads tested.

A clear reduction, and eventual abrogation, of Ad infection of A549 can be observed with increased sugar loading (**Figures 2a, b and c**). Ad infection was blocked at 6000 equivalents of mannose IME (36% total lysine coverage), 12,000 equivalents of galactose IME (73% lysine coverage) and at 6000 equivalents of LeX IME (49% lysine coverage). Relatively, the mannose modification was the most efficient blocking of A549 infection; as infection was abrogated at the lowest percentage coverage.

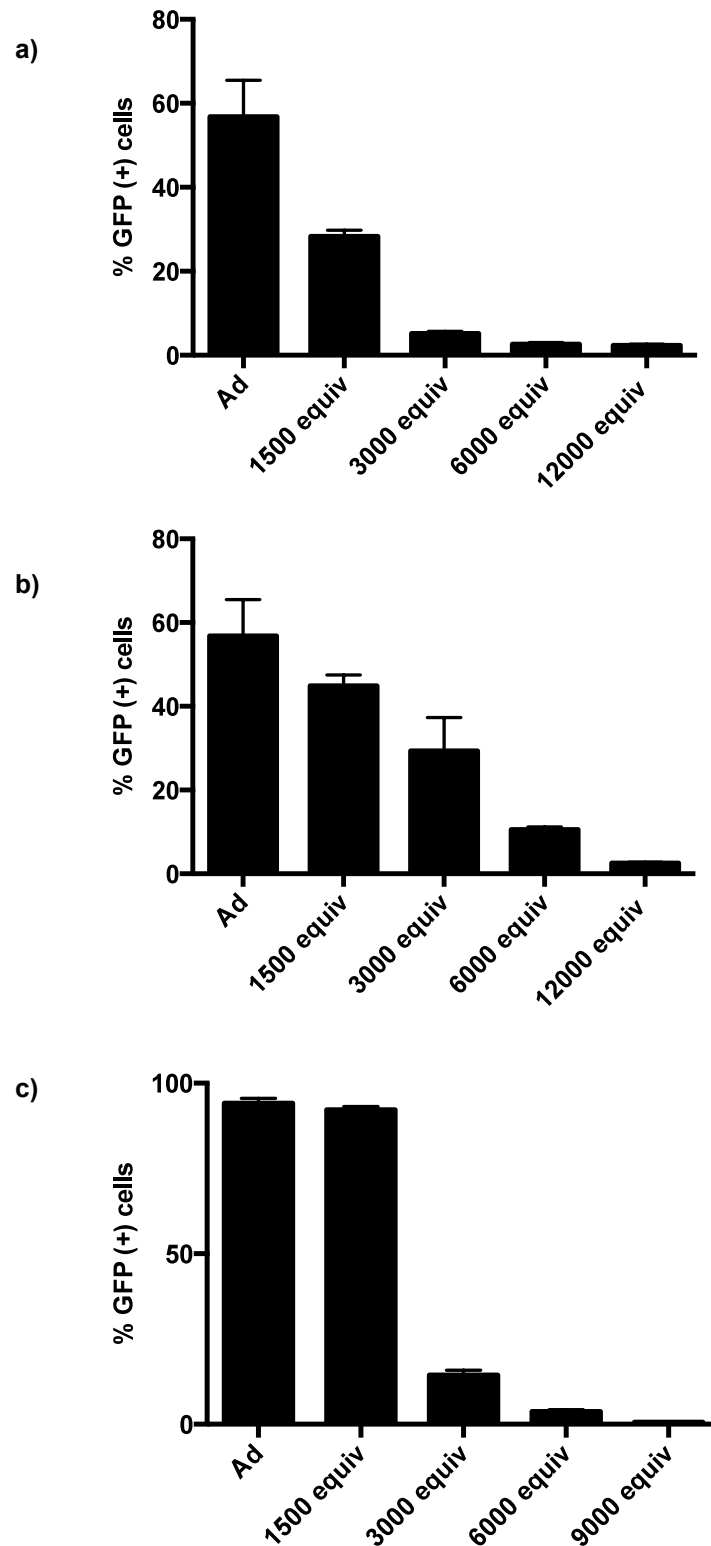


Figure 2 - The infection of CAR+ A549 cells with *gfp*-Ads modified with increasing equivalents of a) mannose, b) galactose or c) Le-X IME reagents. Increasing sugar coating of the viruses decreases the percentage of A549 cells transduced. Percentage of infected cells was measured by flow cytometry. The bars represent the mean of technical triplicates and the error bars are the standard error.

4.2.2 Results: targeting towards MR+ cells

Peripheral blood monocyte cells (PBMCs) can be isolated from human blood samples and subsequently differentiated into other immune cells^{41,42}. Isolation is achieved through separation by Ficoll density gradient and followed by the positive selection of CD14(+) cells. These cells are then incubated in the presence of human cytokines for a number of days.

Initially, an *in vitro* model of 'early macrophages' (3 days M-CSF) was used to assess the infectivity of the glyco-Ads. However, the cells were not well transfected by the naked Ad (5% GFP(+) cells at 1000 MOI) and increasing sugar loading resulted in a decrease of infection (**Figure 3**). The established route of Ad infection involves a two step process; anchoring on the cell surface via CAR interaction followed by binding to integrins, resulting in internalization. Integrins are expressed by cells for cell-cell binding and migration⁴³. PBMC express low levels of integrins owing to their function as a blood component⁴⁴. It was proposed therefore that these early macrophages do not express the required number of integrins in order to facilitate Ad infection. This phenomenon has been noted by Huang *et al*⁴⁵.

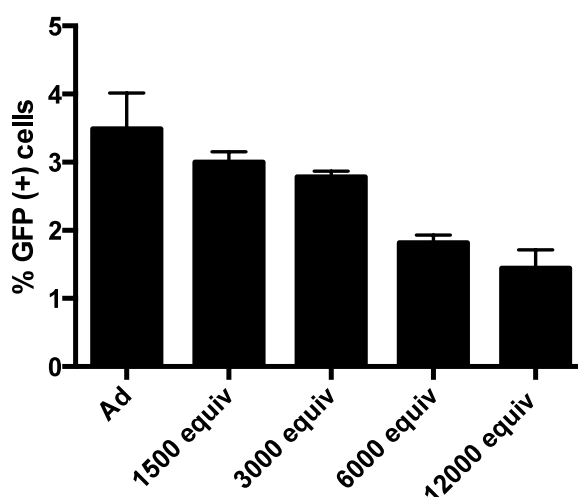


Figure 3 - The infection of PBMC derived early macrophages with Ads modified with increasing equivalents of mannose IME reagent. The early macrophages were resistant to Ad infection and modification with IME mannose decreased levels of infection. The bars represent the mean of technical triplicates and the error bars are the standard error.

PBMCs were differentiated into DC-SIGN(+) late M2 type macrophages (6 days M-CSF, 1 day IL-10 and IL-4), whereupon they became adherent to the plate. These cells were stained for mannose receptor (MR) or DC-SIGN and were found to express both receptors at high levels (**Figure 4**)^{42,46,47}. These macrophages were infected with *gfp*-Ad-Man or *gfp*-Ad-Gal in the presence of media only, media containing either 1% methyl α -D-mannopyranoside or 1% methyl α -D-galactopyranoside. The macrophages were exposed to the virus for 2 hours before being washed into fresh media, in order to avoid any non-specific endocytosis of the Ad particles. 24 hours later infection levels were assessed by the percentage of GFP(+) cells; measured by flow cytometry (**Figure 5**).

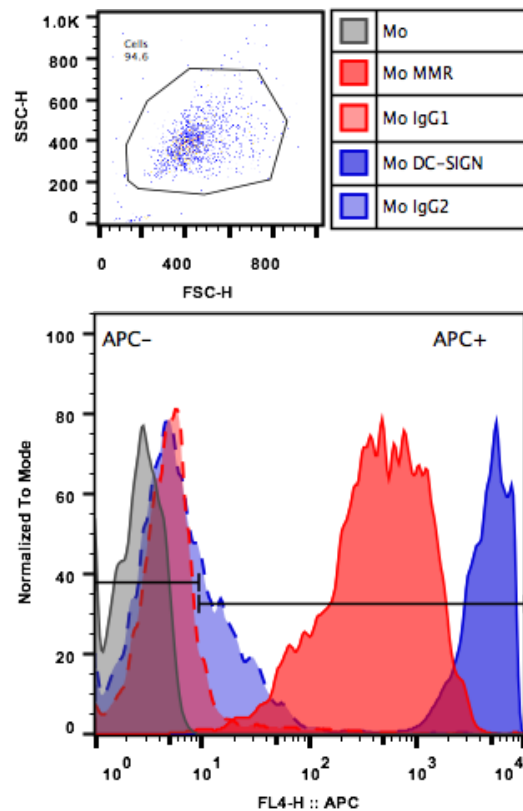


Figure 4 – Fluorescence antibody staining of PBMC derived M2 type macrophages for MR (red) and DC-SIGN (blue). Both receptors are highly expressed. Unstained cells (grey) isotype controls are also shown.

The M2 type macrophages appear to be refractory to Ad infection (~5% of the population) under these conditions. Notably, Ad-Man was 10

times more effective at infecting the macrophages (60% of the population). This level of infection was not significantly improved from 1500-6000 equivalents of IME mannose loading. However, Ad-Man modified with the highest loading of mannose sugar (12,000 equiv, 70% of total lysine coverage) was not able to infect to the same extent, resulting in only around 10% GFP(+) macrophages. It is possible that too much sugar coating might inhibit either the integrin binding to the cell surface or viral endosome escape mechanisms. The increase of infection by the Ad-Man was partially blocked by the addition of a 1% methyl α -D-mannopyranoside solution but not by the 1% methyl α -D-galactopyranoside solution. This suggests that the Ad-Man infection of the macrophages is mannose-specific.

In parallel, the Ad-Gal glycovirus was also used to infect M2 macrophages. Modification of Ad with 1500 equiv of galactose IME sugar (12% total lysine coverage) resulted in a significant increase in infection. This infection level then decreased with increased galactose coverage, with complete abrogation of GFP transduction at 12,000 equivalents IME galactose. Neither the mannose or galactose solutions had any blocking effect on the Ad-Gal infections, suggesting a non-sugar specific infection (see **Figure 6** for a direct comparison of Ad-Man and Ad-Gal infection of macrophages in media only).

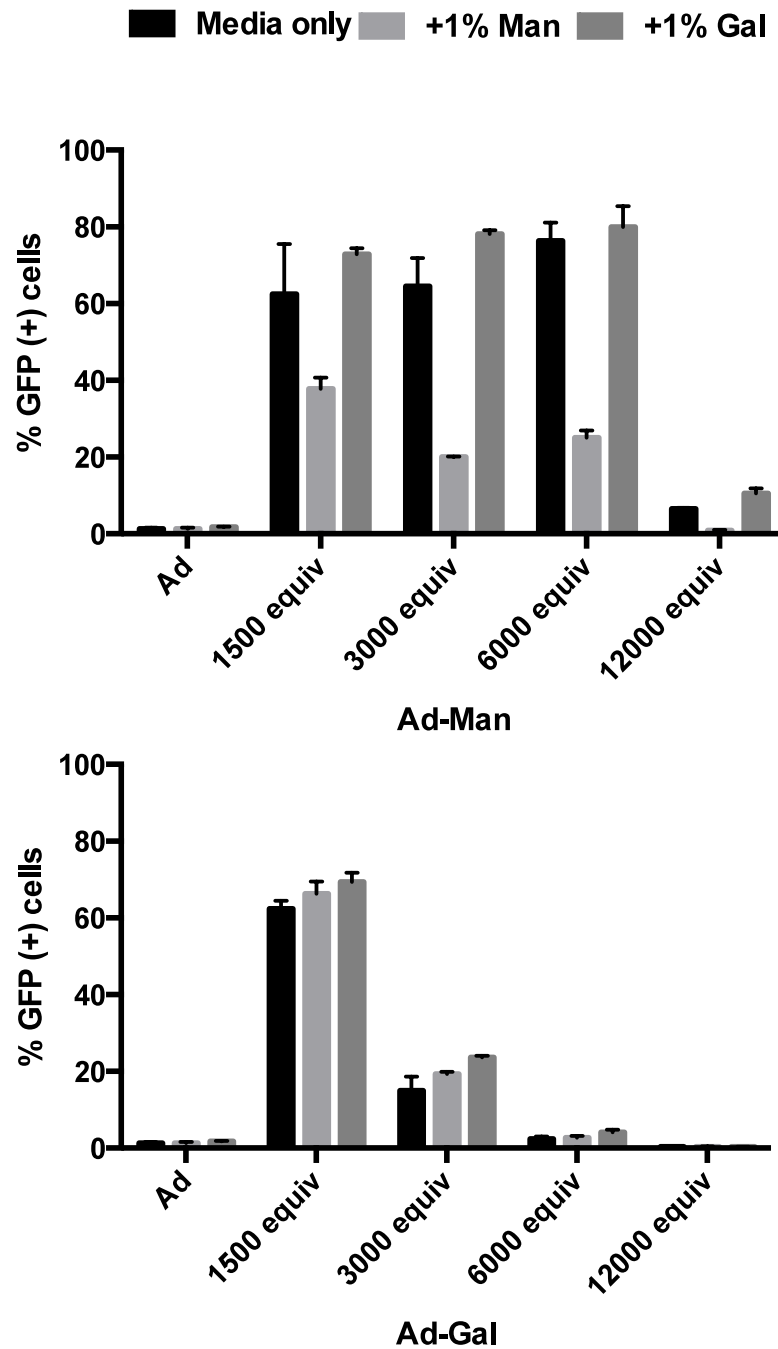


Figure 5 - The infection of MR+ PBMC derived M2 type macrophages with *gfp*-Ad modified with either increasing equivalents of mannose (Ad-Man) or galactose (Ad-Gal) IME reagents. The macrophages were incubated with the Ads for two hours in media only, or media with 1% methyl α -D-mannopyranoside, or 1% methyl α -D-galactopyranoside. The cells were then washed into complete media and the extent of GFP expression was measured by flow cytometry 24 hours later. The bars represent the mean of technical triplicates and the error bars are the standard error.

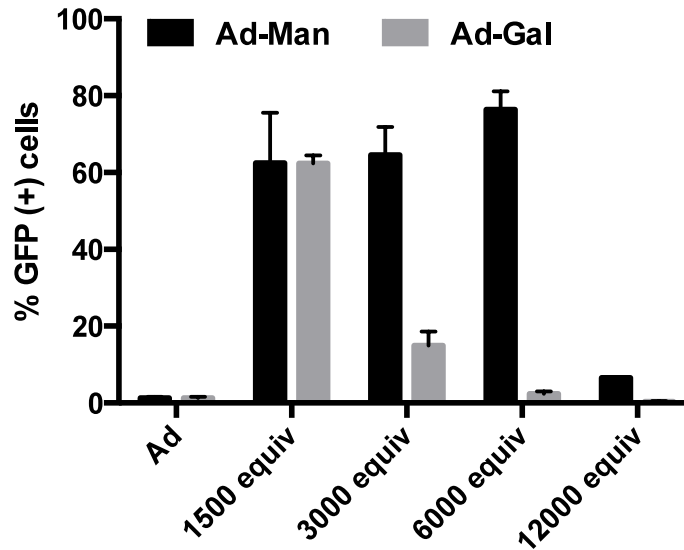


Figure 6 - The infection of MR+ PBMC derived M2 type macrophages with *gfp*-Ad-Man and *gfp*-Ad-Gal modified adenovirus, a direct comparison of the GFP expression resulting from infection in media only. The bars represent the mean of technical triplicates and the error bars are the standard error.

4.2.3 Targeting towards DC-SIGN (+) cells

Although expressed on DC, MR is also expressed on many other cell types⁴⁸. In order to more effectively target only antigen presenting cells *in vivo* a more exclusive receptor was targeted. DC-SIGN is highly expressed on DCs and binds to the trisaccharide Lewis X (LeX)⁴⁹⁻⁵¹. The transgenic DC-SIGN(+) Raji cell line was used as a model to test the DC-SIGN specific transduction capabilities of *gfp*-Ad-LeX (**Figure 7**). The native Raji cells do not express DC-SIGN or MR. Unmodified Ad infected the Raji and Raji DC-SIGN(+) cells similarly, resulting in 30% GFP(+) cells. At the lowest level of LeX modification (1500 equivalents of sugar, 19% total lysine modification) no significant difference between the Raji or Raji DC-SIGN(+) transduction was observed. However, at 3000 equivalents LeX (36% total lysine modification) Raji infection was reduced to ~3% GFP(+) cells whereas 60% of Raji DC-SIGN(+) were GFP(+). At higher sugar coverage the Raji infection was abrogated whilst Raji DC-SIGN(+) infection remained around twice that of unmodified Ad. The extent of the Ad-LeX DC-SIGN(+)

dependent infection also was observed to decrease, to a small extent, over 3000 equivalents of IME sugar.

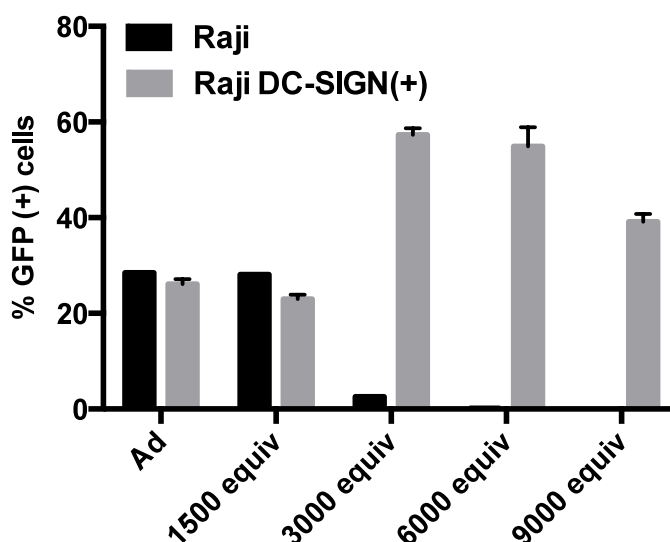


Figure 7 - The infection of DC-SIGN (-) and Tg DC-SIGN(+) Raji cells with *gfp*-Ad modified with increasing equivalents of LeX IME reagent. The bars represent the mean of technical triplicates and the error bars are the standard error.

DCs were derived from PBMC using standard protocols and were stained for expression of MR and DC-SIGN; both receptors were highly expressed (**Figure 8**)⁵². DCs are naturally resistant to Ad infection and high Ad concentrations are required to achieve modest transduction *in vitro*⁵³. As previously observed with macrophage infection, the DCs were only marginally infected by unmodified *gfp*-Ad (~5% cells GFP(+))(**Figure 9**). DCs, from two different blood donors, were infected with *gfp*-Ad-LeX coated with increasing amount of LeX. Notably, Ad-LeX coated at 3000 equivalents resulted in a 16-fold increase in the percentage of infected DCs (80% cells GFP(+)). A slight decline in infection at 6000 to 9000 equivalents of LeX was observed, reflecting the trend seen in the infection of the Raji DC-SIGN(+). It is possible that excess sugar coating might inhibit either the integrin binding to the cell surface or other viral transduction mechanisms.

Data from **Figure 9** and **Figure 4** has been combined in **Figure 10**, allowing visualisation of the switch in Ad tropism towards the DC-SIGN(+) DCs. The Ad-LeX at 3000 equivalents of sugar coating (36% total lysine coverage) yields the highest levels of GFP(+) DCs. This level of LeX coating was used throughout the all the subsequent experiments.

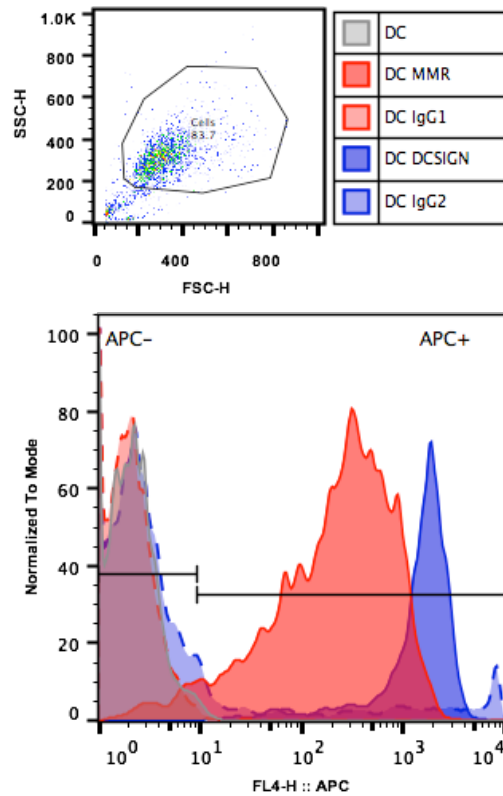


Figure 8 – Fluorescent antibody staining of PBMC derived DCs for MR (red) and DC-SIGN (blue). Both receptors are highly expressed. Unstained cells (grey) isotype controls are also shown.

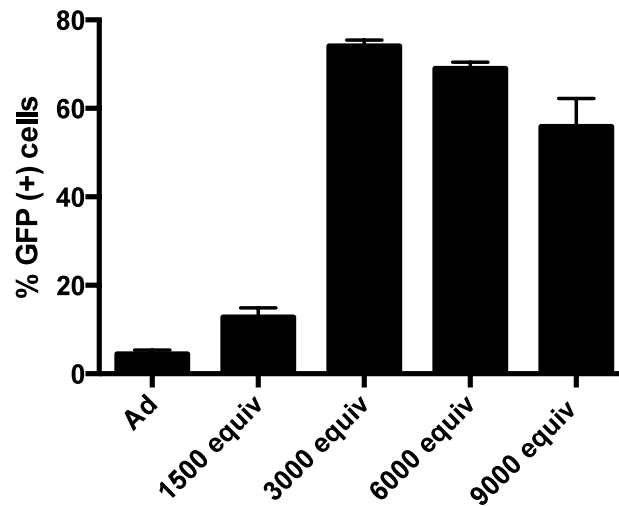


Figure 9 - The infection of DC-SIGN(+) PBMC derived DCs with Ads modified with increasing equivalents of LeX. The DCs were incubated with Ads for two hours in media, the cells were then washed into complete media and the extent of GFP expression was measured by flow cytometry 24 hours later. Bars represent the average of two blood donors performed in technical triplicate. Error bars are the standard error.

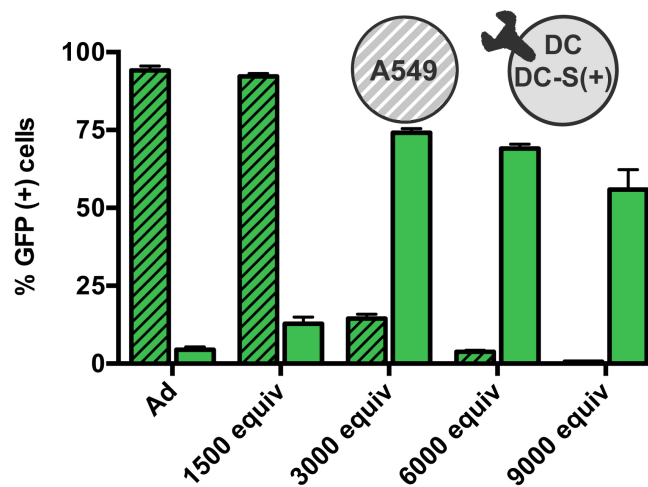


Figure 10 - The infection of CAR(+) A549 or DC-SIGN(+) PBMC derived DCs or A549s with Ads modified with increasing equivalents of LeX. The cells were incubated with the Ads for two hours in media, the cells were then washed into complete media and the extent of GFP expression was measured by flow cytometry 24 hours later. Direct comparison of the relative infection percentages, data drawn from figures 4 and 9.

Following successful demonstration of tropism switching towards DC-SIGN(+) DCs, a blocking experiment was conducted to verify the DC-SIGN dependence of the novel infection route of the Ad-LeX. A DC-SIGN blocking antibody (AZN-D1) has been developed by Geijtenbeek *et al* and was used to block human DCs, prior to exposure to the Ad-LeX⁵⁴. The presence of the blocking antibody did not affect the unmodified Ad infectivity, however, the antibody did successfully reduce the infection of Ad-LeX, confirming that infection with Ad-LeX is, at least in part, mediated by DC-SIGN (Figure 11).

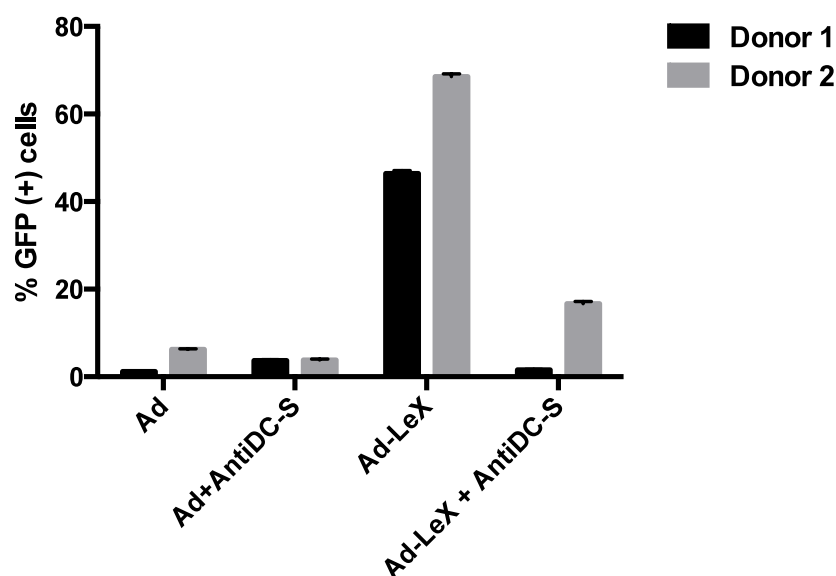


Figure 11 - PBMC derived human DCs, from two blood donors, were infected with Ad-LeX (modified with 3000 equivalents of LeX IME sugar) at 1000 MOI. DC-SIGN blocking was achieved with anti-DC-SIGN antibody⁵⁴. Ad-LeX infection of DCs is significantly reduced in the presence of the blocking antibody. The bars represent the mean of technical triplicates and the error bars are the standard error.

PBMC-derived human DCs, from two different blood donors, were infected with increasing concentrations of Ad or Ad-LeX (Figure 12). Apoptosis of the cells was not observed at any concentration of virus. Increasing concentrations of Ad-LeX resulted in increasing proportions of GFP(+) cells in both donors, with up to 98% of DC infected at 1000 MOI. A slight increase in the infection of unmodified Ad was observed with

increasing concentration, however only a maximum of 18% of DCs were GFP(+).

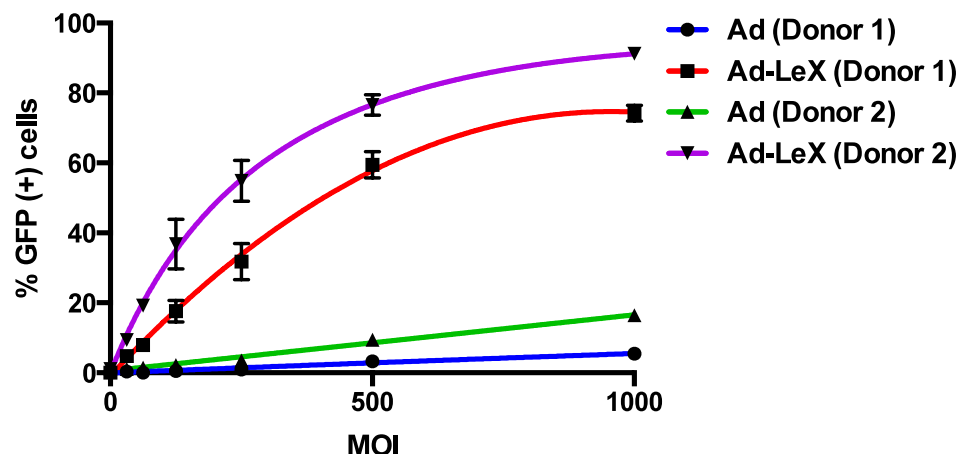


Figure 12 - Concentration dependence of *gfp*-Ad-LeX infection of PBMC derived DCs. Infection Ad-LeX results in a greater % of GFP(+) DCs at all concentrations of virus. Averaged over two blood donors. The data points represent the mean of technical triplicates and the error bars are the standard error.

Blood coagulation factor X (FX) binds to the hexon protein of the Ad capsid with an affinity in the nanomolar range³⁸. Once bound, the FX acts as a bridging ligand between the Ad and HSGAGs present on hepatocytes. This interaction results in enhanced Ad internalization into hepatocytes; such a large influx of virus can cause toxicity *in vivo*²⁷. As the hexon-FX interface involves lysine residues it was investigated whether the LeX IME sugar modification might prevent the FX-dependent infection. Ad was incubated at 37°C with purified human FX before being used to infect HepG2 cells (MOI 50). The HepG2 were found to be very susceptible to Ad infection, even at low MOI, without FX (**Figure 13**). As such, all cells were GFP(+) and it was therefore more meaningful to represent the overall infection by the mean fluorescence intensity (MFI) of GFP. Intriguingly, infection with Ad-LeX resulted in significantly greater MFI of GFP than unmodified Ad (**Figure 13**). In agreement with the literature Ad+FX was able to infect the HepG2 cells over 75 fold more efficiently compared to Ad

alone (**Figure 14**)^{19,20,21}. The level of infection from Ad-LeX+FX however was 30 fold less than that of Ad+FX, suggesting an abrogation of the capsid-FX interaction. Residual FX dependent enhancement of Ad-LeX infection, around 2 fold (**Figure 15**), was observed but the decrease, relative to Ad+FX, is striking.

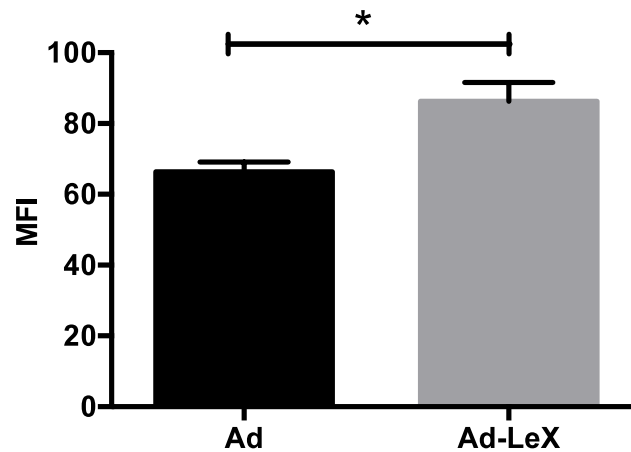


Figure 13 - The infection of HepG2 cells with Ad or Ad-LeX at 50 MOI. Ad-LeX infection results in significantly more GFP expression than Ad infection. Cells were exposed to the virus for 2 hours before being washed into complete media. 24 hours later levels of infection were assessed by the mean fluorescence intensity (MFI) of GFP expression, measured by flow cytometry. The bars represent the mean of technical triplicates and the error bars are the standard error. Statistics performed was an unpaired T-test where * p <0.05.

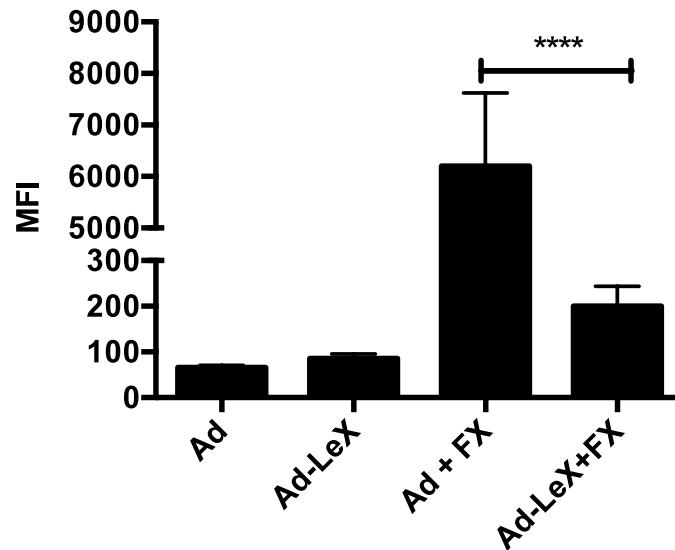


Figure 14 - Ad and Ad-LeX were incubated with purified human FX before being used to infect HepG2 cells, at 50 MOI. The Ad-LeX-FX interaction results in significantly less GFP expression compared to Ad-FX. Cells were exposed to the virus for 2 hours before being washed into complete media. 24 hours later, levels of infection were assessed by the mean fluorescence intensity of GFP expression, measured by flow cytometry. The data points represent the mean of technical triplicates and the error bars are the standard error. Statistics performed was an unpaired T-test where * $p < 0.05$.

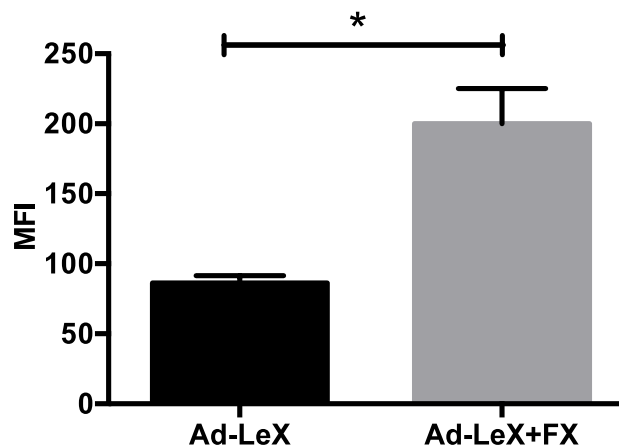


Figure 15 - Ad-LeX was incubated with purified human FX before being used to infect HepG2 cells, at 50 MOI. Cells were exposed to the virus for 2 hours before being washed into complete media. 24 hours later levels of infection were assessed by the mean fluorescence intensity of GFP expression, measured by flow cytometry. The data points represent the mean of technical triplicates and the error bars are the standard error. Statistics performed was an unpaired T-test where * $p < 0.05$.

70% of the human population carries anti-Ad5 antibodies- a fact which represents a major obstacle to the widespread use of Ad DNA vaccine vectors^{55,56}. Antibodies can directly neutralise Ad, blocking cell receptor sites and preventing viral un-coating, and are also handles for complement opsonisation and clearance (**Figure 1**). To investigate whether the LeX modification might shield the virus from antibody neutralization, inhibition cell infection assays were conducted. As the tropism of Ad had been switched from CAR(+) cells towards DC-SIGN(+) cells there was no cell line for which the infection level of unmodified Ad and Ad-LeX was comparable. Therefore antibody neutralisation of Ad was analysed in Raji or A549 and the neutralisation of Ad-LeX, in DC-SIGN(+) Raji or human DCs. Firstly, anti-Ad-5 rabbit serum was used in an inhibition study. In brief, the viruses were incubated in varying dilutions of rabbit serum at 37°C before being used to infect A549 or DCs. After 24 hours the level of infection was assessed by the percentage of GFP(+) cells, as measured by flow cytometry. 0% inhibition was set as the proportion of GFP(+) cells, resulting from Ad or Ad-LeX infection, that had not been exposed to serum. A rabbit serum titration was also conducted on Ad infecting Raji cells to ascertain neutralisation levels (**Figure 16**).

When the neutralisation of Ad and Ad-LeX in Raji and Raji DC-SIGN (+) cells were compared the IC₅₀ values were near-identical (Ad 1.34×10^{-4} and Ad-LeX 1.42×10^{-4}), indicating that the sugar modification had no effect on antibody binding (**Figure 17**). Further investigation into the molecular mechanisms of Ad5 neutralisation revealed that rodent and human antibodies are directed at different Ad capsid proteins^{57,58}. The use of rabbit serum therefore was not a good model for neutralisation studies of our glyco-Ads.

Human blood serum was isolated from five blood donors and tested for anti-Ad5 reactivity by ELISA (**Figure 18**). All donors displayed reactivity however, one donor (Donor 5) had a particularly strong response- this serum was chosen for the subsequent *in vitro* neutralization

experiments. A comparison between the rabbit and human serum was performed (Figure 19). The rabbit serum displayed significantly stronger binding and neutralization than the tested human serum.

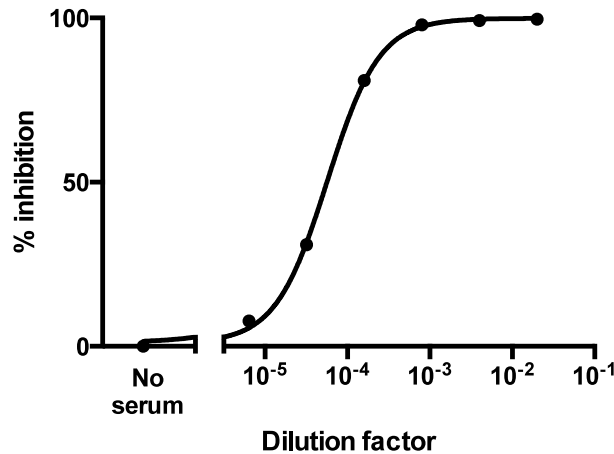


Figure 16 - Inhibition assay of *gfp*-Ad infection of A549 cells after viruses had been incubated with increasing dilutions of anti-Ad5 rabbit serum. A pilot experiment to ascertain the IC₅₀ of the rabbit serum. 0% inhibition was set as the proportion of GFP(+) cells resulting from Ad or Ad-LeX that had not been exposed to serum.

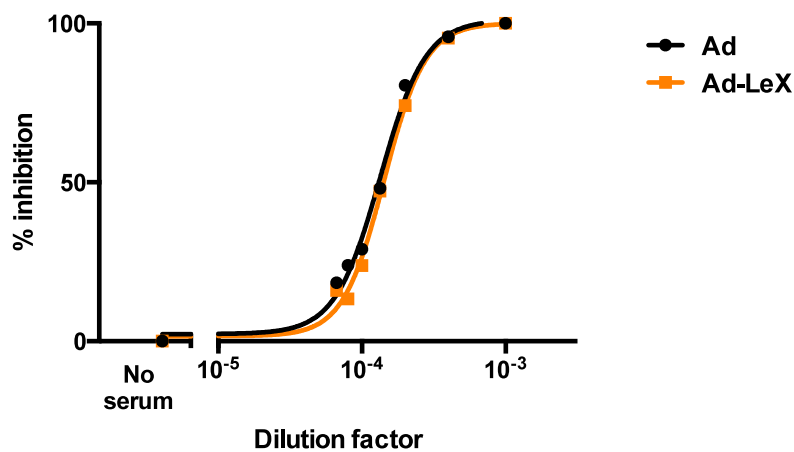


Figure 17 - *gfp*-Ad and *gfp*-Ad-LeX were incubated with dilutions of anti-Ad5 rabbit serum. Ad was used infect A549 and Ad-LeX was used to infect DC-SIGN (+) Raji. After 24 hours the extent of infection was assessed by the percentage of GFP(+) cells, measured by flow cytometry. 0% inhibition was set as the proportion of GFP(+) cells resulting from Ad or Ad-LeX that had not been exposed to serum. The data points represent the mean of technical triplicates and the error bars are the standard error.

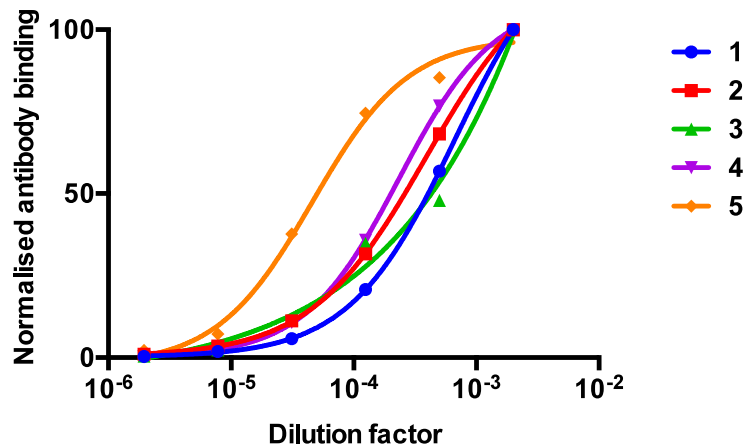


Figure 18 - IgG antibody reactivity of human sera against Ad-5. An ELISA plate was coated with unmodified Ad5 and sera from five different blood donors were tested for anti-Ad antibody. Blood donor 5 shows the greatest response against Ad.

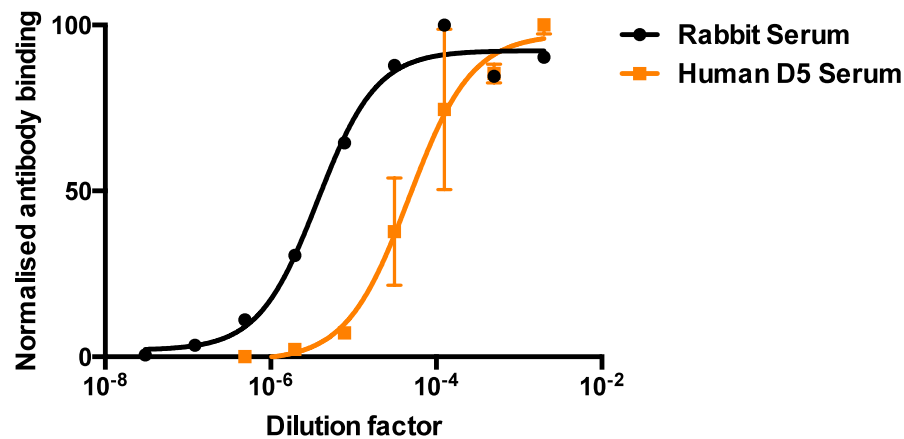


Figure 19 - Comparison of the reactivity of rabbit and human sera against Ad-5. An ELISA plate was coated with unmodified Ad5 and serum from an immunized rabbit (Adcam) and serum from donor 5 (Figure 18) were tested for anti-Ad antibody at decreasing concentration. Rabbit serum is more reactive against Ad than the human serum. The data points represent the mean of technical triplicates and the error bars are the standard error.

An identical neutralization experiment, infecting A549 and human DCs, was then performed using human blood serum (Figure 20). The IC₅₀ for Ad infection of A549 was calculated at a dilution of 0.0252 and for the Ad-LeX infection of DCs was 0.0866. The *gfp*-Ad-LeX was significantly

($P > 0.0001$) better able to transduce the target cells in the presence of human serum, compared to the unmodified Ad. The chemical glycosylation of the Ad capsid with the Lewis X trisaccharide therefore reduces the neutralisation potential of human serum.

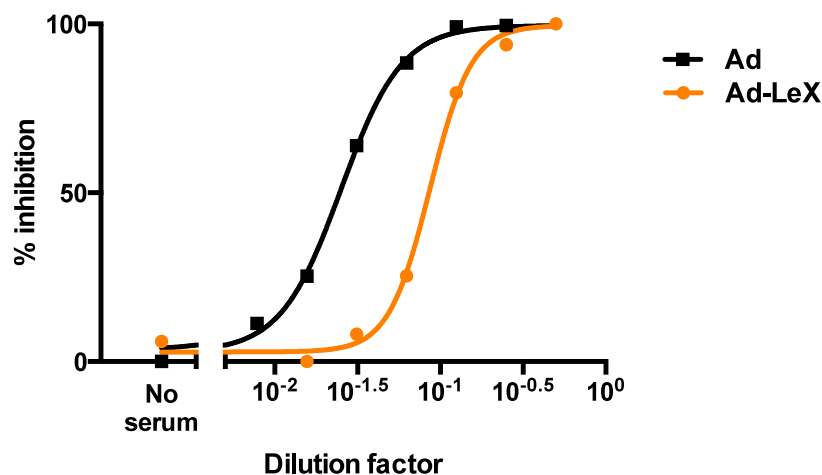


Figure 20 - *gfp*-Ad and *gfp*-Ad-LeX were incubated with dilutions serum from blood donor 5 (Figure 18), Ad was used to infect A549 and Ad-LeX was used to infect human PBMC derived DCs. After 24 hours the extent of infection was assessed by the percentage of GFP(+) cells, measured by flow cytometry. 0% inhibition was set as the proportion of GFP(+) cells resulting from Ad or Ad-LeX that had not been exposed to serum. The data points represent the mean of technical triplicates and the error bars are the standard error.

4.3 Discussion

The direct modification of Ad capsid lysine residues with mannose, galactose, and LeX IME sugars allows for lectin-specific switching of viral tropism. Infection of A549 cells CAR(+), naturally susceptible to Ad infection, was reduced and abrogated with increased levels of sugar loading. Mannose-modified glyco-Ads were able to infect M2 type macrophages, in a mannose specific manner, 10-fold more efficiently than naked Ad. Similarly, LeX modified glyco-Ads infected human DCs with 10-fold greater efficiency than Ad; an enhancement blocked by anti-DC-SIGN antibody. The LeX modification was also demonstrated to shield the Ad

from two important Ad sequestration mechanisms present *in vivo*. The FX-dependent enhancement of Ad infection in hepatocytes was blocked and a 4- fold greater titre of human sera was required to neutralise Ad-LeX, relative to Ad. This is the first successful instance of shielding Ad from anti-Ad sera using carbohydrates. Mannosylation of Ad by Espenlaub *et al* did not achieve lectin targeting nor anti-Ad antibody shielding⁵⁹. This tropism switch towards therapeutically relevant cells and protection from host sequestration achieved by the Ad-LeX promises to yield a more effective and safer DNA vaccine vector. In comparison to previous attempts to modify Ad, the IME sugar approach offers a one-pot, single step, solution to the problems of Ad promiscuity, toxicity and neutralisation *in vivo*. It was not established whether the glyco-Ads were internalising by the new, targeted lectin (MR or DC-SIGN) or whether they used the lectin to anchor to the cell surface, in the same manner that unmodified Ad uses CAR. The glyco-Ads could also be internalising via the regular integrin pathway. This could explain the observation that at the highest levels of sugar loading, infection levels were shown to decrease. For example, Ad-Man, reacted with 12,000 equivalents of IME, infected macrophages 10 times less than Ad-Man at 6,000 equiv IME. In order to test this theory infection studies should be conducted, whilst blocking integrins through the addition of excess RDG peptides.

4.4 Experimental

Cell culture and isolation. A549 and HepG2 cells were maintained in complete DMEM medium (heat inactivated 10% fetal calf serum, 2 mM L-glutamine, 100 g/mL streptomycin and 100 U/mL penicillin). Raji, PMBC derived DCs and macrophages were maintained in complete RPMI medium (10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100g/mL streptomycin and 100 U/mL penicillin). Peripheral blood mononuclear cells (PBMCs) were extracted from lymphocyte cones and differentiated into conventional dendritic cells (DCs). The lymphocyte cones were drained and

lymphocytes separated on a Ficoll (GE healthcare) gradient. These cells were extracted and washed extensively; any remaining erythrocytes were lysed with lysis buffer (Gibco). CD14(+) cells were further separated using positive selection MACS® beads (Miltenyibiotec), counted and plated (5×10^4 per well) in complete RPMI containing 100ng/mL IL-4 and 50ng/mL GM-CSF (Biolegend). The DCs were cultured for a total of 6-9 days before infection, with an exchange of media every 3 days⁶⁰. PBMCs were also differentiated into early macrophages by incubation with 100ng/mL M-CSF for 3 days in complete media, or late M2 macrophages by incubation with 50ng/mL M-CSF for 6 days plus 24 hours with the addition of 20ng/mL IL-4 and IL-10⁴⁷ (Biolegend).

Receptor staining. Cells were washed and transferred into staining buffer (PBS, 2% BSA) and split 1×10^4 cells/50 μ L/well into a V bottomed 96 well plate. DCs and macrophages were incubated with Fc block (BDBiosciences) for 15 minutes at 4°C prior to staining. The staining antibodies (Biolegend) were then added at the dilutions recommended by the manufacturer and the plate was shaken gently at 4°C, protected from light, for 30 minutes. The cells were centrifuged in the plate (400xg, 3min 4°C) and washed four times with staining buffer (200 μ L). The cells were analysed for fluorescence by flow cytometry (Becton Dickinson FACSCalibur four colour, two laser).

Infection of A549. A549 cells were plated at 5×10^4 cell/well/100 μ L in flat bottomed 96 well plates. The cells were allowed to adhere for several hours before infection with adenovirus expressing GFP (*gfp*-Ad), modified with mannose (*gfp*-Ad-Man) or galactose (*gfp*-Ad-Gal) or Le-X (*gfp*-Ad-LeX) at 100 MOI. The viruses were diluted in PBS to a final volume of 10 μ L and were then added to the wells. After 2 hours the viruses were washed off cells with complete DMEM medium. The cells were left for a further 24hrs, before transduction was detected by GFP expression using flow cytometry analysis. A549 cells were lifted from the plate by trypsin digest.

Infection of early and late macrophages. PBMC derived human macrophages were infected with adenovirus expressing GFP (*gfp*-Ad), modified with mannose (*gfp*-Ad-Man) or galactose (*gfp*-Ad-Gal) at 1000 MOI. The viruses were diluted in PBS to a final volume of 10 μ L and were then added to the wells. After 2 hours, the viruses were washed off the cells with complete RPMI medium and the macrophages were left for a further 24hrs, before transduction was detected by GFP expression using flow cytometry analysis. Blocking was achieved by prior incubation of the cells with 1% methyl α -D-mannopyranoside or 1% methyl α -D-galactopyranoside.

Infection of Raji and Raji DC-SIGN(+) cells. Raji and transgenic Raji DC-SIGN (NIH AIDS Reagent Program) cells were infected with adenovirus expressing GFP (*gfp*-Ad) or adenovirus modified with LeX (*gfp*-Ad-LeX) at 1000 MOI. The viruses were diluted in PBS to a final volume of 10 μ L and were then added to the wells. After 2 hours, the viruses were washed off the cells with complete RPMI medium and the DCs were left for a further 24hrs, before transduction was detected by GFP expression using flow cytometry analysis.

Infection of dendritic cells (DCs). PBMC derived human DCs were infected with adenovirus expressing GFP (*gfp*-Ad) or adenovirus modified with LeX (*gfp*-Ad-LeX) at 100 MOI. The viruses were diluted in PBS to a final volume of 10 μ L and were then added to the wells. After 2 hours, the viruses were washed off the cells with complete RPMI medium and the DCs were left for a further 24hrs, before transduction was detected by GFP expression using flow cytometry analysis. Blocking was achieved by prior incubation with the anti-DC-SIGN blocking monoclonal antibody AZN-D1 (20 μ L/mL)⁵⁴.

Transfection HepG2. Ads were diluted to 100MOI/10 μ L in PBS and were opsonized in 10 μ g/mL human FX (Haematologic technologies) for 1 hour

at 37°C. HepG2 cell were plated at 2.5×10^4 cells per well in 100µL of serum free DMEM media and allowed to adhere. The Ads were then added and left on the cells for 2 hours before the cells were washed into complete DMEM. After 24 hours, the cells were removed with enzyme free dissociation buffer (Gibco) and analyzed for GFP expression with flow cytometry.

Antibody binding assay. Whole human blood was collected, left for 1 hour to coagulate, and then centrifuged to isolate the sera (3,000 rpm, 10 min 4°C). Standard ELISA plates were coated over night at 4°C with unmodified Ad5 (1×10^9 vp/50µL/well) diluted in 50mM carbonate-bicarbonate buffer pH 8.5. Plates were then washed with washing buffer three times (PBS, 0.05% tween). The plates were then blocked for 1 hour at room temperature (RT) with SuperBlock 50µL (Thermo Fisher). KLH protein (10ug/mL) was used as a control. Serial dilutions of human sera or anti-Ad5 rabbit serum (Abcam) were added to the plate (50µL) for 2 hours at RT. Plates were then washed with washing buffer three times. The IgG bound fraction was then detected with anti human IgG-horse radish peroxidase (HRP) for 1 hour and then washed. Finally, the amount of binding was visualized by the addition of 50µL TMB substrate. After 5-10 minutes, in the dark, a blue color developed and the reaction was stopped (50µL 1M HCl). Absorbance was then measured at 420nm.

Human serum inhibition assay. Ad and Ad-LeX samples were incubated (37°C, 1 hour) with decreasing concentrations of rabbit anti-Ad5 serum (Abcam) or human serum in PBS. The Ad and Ad-LeX samples were then used to infect either A549 cells or PBMC derived human DCs (in 0% FCS), respectively. The MOI of viruses used was adjusted to result in a similar level of infection at 0% serum (200 MOI for Ad on the A549 and 500 MOI for Ad-LeX on the DCs). The volume of the serum dilutions used to pre-incubate the viruses was adjusted to take into account the difference in the ratio of serum/virus particles in the two samples. The viruses were left on

the cells for 4 hours before the cells were washed into complete RPMI media. After 24 hours, cells were analyzed for GFP expression by flow cytometry. The percentage of GFP(+) cells was normalized and plotted against the dilution factor of the serum.

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5. DC immunology

5.1 Introduction

5.1.1 Co-stimulatory molecules and cytokines

Antigen presenting cells (APCs), in particular dendritic cells (DCs), are described as the sentinels of the immune systems. APCs capture and process the proteins they encounter and present their findings to T cells in the lymph nodes. During the capture process, APCs are stimulated through the interaction of toll-like-receptors (TLRs) with pathogen associated molecular patterns (PAMPs)^{1,2}. Depending on which PAMPs are encountered, the APC will up-regulate certain co-stimulatory molecules and secrete certain cytokines; communicating information on the type of antigen captured. The collective nature of these signals is described as the mature DC phenotype. Naïve T cells receive this signal during the binding event between the MHC-antigen-peptide-complex and the T-cell receptor (TCR). The co-stimulatory molecules and cytokines from the APC inform any subsequent T cell proliferation and differentiation (**Figure 1**)².

TLRs exist both on the cell surface and within the cytosol of the APCs, with each TLR specific for a particular PAMP². Intracellular TLRs tend to bind to PAMPs associated with viral infection. TLR-9 is particularly relevant to Ad, and to the use of Ad as a DNA vaccine vector. This TLR binds to and is activated by non-methylated CpG oligo-deoxynucleotides, which are abundant in microbial genomes but rare in vertebrate genomes^{3,4}. The Ad genome contains several non-methylated CpG motifs, which are the source of the DC maturation upon Ad infection^{5,6}.

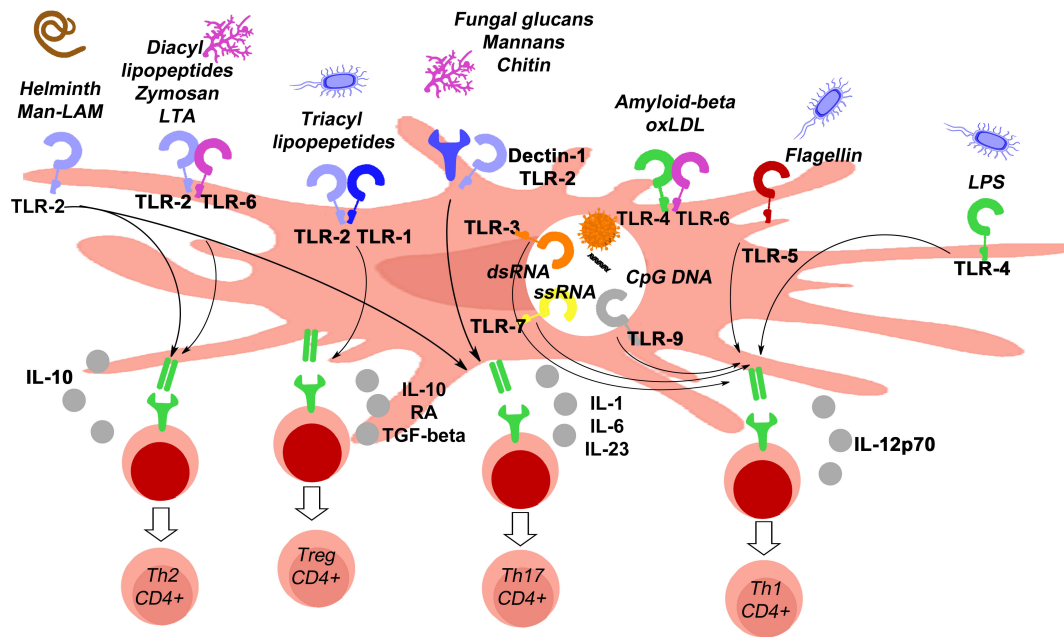


Figure 1 - Toll-like-receptors (TLRs) on DCs interact with pathogen associated molecular patterns (PAMPs), causing the DC to mature and secrete particular cytokines. These cytokines influence the differentiation of naïve T cells upon the MHC-TCR binding event^{2,7,8}.

In order to generate an effective DNA vaccine, the transgene must be transcribed and translated endogenously, and be presented to T cells along with co-stimulatory molecules and cytokines. Previously, it has been shown that the tropism of Ad-LeX was successfully switched to infect DC-SIGN(+) DCs. It was next important to evaluate whether i) the transgene is being processed and presented to T cells and ii) DCs are stimulated by the innate antigenicity of the Ad.

5.1.2. Immune modulation resulting from lectin-TLR cross talk

Recent work has demonstrated that certain lectins, including DC-SIGN, possess the ability to *modulate* signals from TLRs^{9,10,11}. DC-SIGN has demonstrated particular plasticity in modulating TLR-4 signaling and is able to tune towards a pro- or anti-inflammatory response⁹. In response to distinct DC-SIGN-pathogen interactions DCs can promote or inhibit the proliferation of T helper type I (Th1), T helper type 2 (Th2) or regulatory T

cells (Tregs)¹²⁻¹⁵. The expression of pro-inflammatory cytokines, in response to LPS (a TLR-4 ligand), is enhanced in the presence of mannose^{16,17}. Mechanistically, interaction with mannose-containing carbohydrates results in the phosphorylation of Raf-1, an event that leads to increased transcription and secretion of IL-12 and IL-6. By contrast, interaction of DC-SIGN with fucose-containing sugars, including LeX, elicits the secretion of IL-10 and the down-regulation of IL-12 and IL-6 from DCs, effectively suppressing the response to LPS¹⁸.

Artificial carbohydrate structures conjugated to a vaccine vector could therefore have a dual role, not only as targeting ligands but also as mimics of or 'pseudo'-PAMPs. The possibility that Lewis-X modification of the Ad capsid could alter, and thus potentially tune, the immune response from DCs was therefore investigated.

Table 1 - Lectins expressed on dendritic cells, the binding ligands and subsequent immune modulation effects¹⁹⁻²². Abbreviations: Leukotrine B 4 (LTB₄); T helper type 1 or 2 (Th1 or Th2); toll like receptor (TLR); mucin-2 (MUC-2); mannosylated lipoarabinomannan (manLAM), α -N-acetylgalactosamine (GlcNAc); α GalNAc-Ser/Thr (Tn antigen); GalNAc β 1-4GlcNAc (LacdiNAc epitope).

C-type-lectin	Expression	Known glycan PAMP	Immunological outcome
DC-SIGN	Myeloid DCs	High mannose Fucose, LeX, LeY, LeA, LeB	<i>Mannose = Up-regulation of TLR-induced IL-12, IL-10 and IL-6 production Fucose, Le-X = Up-regulation of TLR-induced IL-10 production and inhibition of IL-12 and IL-6</i>
Langerin (CLEC4K, CD207)	Langerhans cells Dermal DCs	High Mannose Fucose, LeY, LeB GlcNAc	<i>Endocytosis and antigen presentation</i>
MGL (CLEC10a,CD301)	Myeloid DCs Macrophages	Terminal GalNAc Tn antigen, LDN	<i>ND</i>
Dectin 1 (CLEC7A)	Myeloid DCs Monocytes Macrophages B cells	β -1,3-glucan	<i>Th1 and Th17 differentiation through induction of IL-1 beta, IL-6, IL-12 and IL-23 production. Induction of TNF and CXCL2 production. LTB₄ synthesis. Involved in MUC-2 induced anti-inflammatory complex</i>

			<i>formation on DCs</i> ²³
MICL (CLEC12A, DCAL2)	Myeloid DCs Monocytes Macrophages Neutrophils	ND	<i>Inhibition of TLR-4-induced IL-12 production</i>
DNCR1 (CLEC9A)	BDCA3(+) DCs, Monocytes B cells	F-actin exposed on necrotic cells ^{24 25}	<i>Induction of TNF production Antigen cross-presentation</i> ^{26 27}
Dectin 2 (CLEC6A)	Myeloid DCs Plasmacytoid DCs Macrophages B cells Neutrophils	α -mannose Mannobiose-rich residues ManLAM ²⁸	<i>Induction of TNF and IL-6 production. Cysteinyl leukotriene synthesis. Th17-biased immunity in response to fungi</i> ^{29,30} <i>ManLAM=Th17 response</i> ²⁸
BDCA2 (CLEC4C, CD303)	Plasmacytoid DCs Monocytes Macrophages Neutrophils	ND	<i>Inhibition of TLR-9-induced type I IFN, TNF and IL-6 production. Up-regulation of TLR-9 induced IL-10 production Induction of TNF, IL-10 and CXCL2 production.</i>
MINCLE (CLEC4E)	Myeloid DCs Monocytes Macrophages	α -mannose Mycobacteria trehalose dimycolate ³¹	<i>Mycobacteria trehalose dimycolate=Th17 promoting</i> ³² <i>Fungal engagement suppresses Dectin-1.</i> ³³
DCIR (CLEC4A)	Myeloid DCs Plasmacytoid DCs Monocytes Macrophages B cells Neutrophils	ND	<i>Inhibition of TLR-8-induction. TNF and IL-12 production. Inhibition of TLR-9-induced TNF and IFN alpha production</i>
Mannose receptor (CD206)	Myeloid DCs Macrophages	High mannose, fucose Sulphated-LeX	<i>Phagocytosis and antigen presentation</i>
DEC205 (Ly75, CD205)	Myeloid DCs	ND	<i>Endocytosis and antigen presentation</i>
Macrophage galactose lectin (MGL)	Immature and tolerogenic DCs, macrophages, dermal CD1a DCs, blood CD1c myeloid DCs	Galactose Terminal GlcNAc Sialylated and non-sialylated Tn antigen LacdiNAc epitope (GalNAc β 1-4GlcNAc) ³⁴⁻³⁷	<i>Induces DC maturation and antigen presentation</i> ^{38,39} <i>Augments TLR-2 responses enhancing IL-10 and TNF-α</i> ⁴⁰

5.1.3 Transgenic DC-SIGN(+) mice

Mice do not express DC-SIGN but instead express up to eight murine homologs, which do not share the same sugar-binding partners⁹. Mouse mSIGNR-3 shows binding to the Lewis sugars, with greatest affinity for Le-A and least for LeX⁴¹. SIGNR3, the closest sequence homologue, is also the

only homologue that, like human DC-SIGN, possesses dual specificity for glycans with high mannose and fucose content. mSIGNR-1, the second-closest homolog to human DC-SIGN, has been shown to bind α -mannose monosaccharides but not the Lewis series⁴². As mSIGNR-3 does not display the same sugar binding profile as hDC-SIGN, it would not be an accurate model for targeting studies with Ad-LeX.

A transgenic C57BL/6 mouse line was developed by Sparwasser *et al*⁴³. A transgene for human DC-SIGN was introduced under the control of a CD11c minimal promoter. This model allows for the study of hDC-SIGN targeting for the induction of CD4(+) and CD8(+) T cell responses. Bone-marrow-derived DCs (BMDCs) from tg hDC-SIGN mice were able to induce the proliferation of both CD4(+) and CD8(+) T cells *in vitro*, illustrating that hDC-SIGN is able to endocytose, and cross-present, in the mouse DC model^{44,45}. Thus, these tg hDC-SIGN mice were used to study the targeting of Ad-LeX both *in vitro* and *vivo*. The ability of the Ad-LeX infected tg hDC-SIGN mouse BMDCs to promote T cell proliferation was also investigated.

5.2 Results

5.2.1 Human dendritic cell (DC) stimulation

Human PBMC-derived DCs (from four different donors) were infected with *gfp*-Ad or *gfp*-Ad-LeX. 24 hours later, the DCs were stained with fluorescent antibodies for the canonical co-stimulatory molecules. The extent of co-stimulatory molecule up-regulation was then assessed by flow cytometry by measurement of the mean intensity of fluorescence (MFI). Samples of the supernatant from the DCs were also extracted and cytokine concentrations were measured by Luminex® assay. During the post-acquisition analysis of the flow cytometry data, the MFI was measured for the whole DC population, then from GFP(-), uninfected DCs alone and GFP(+), Ad infected DCs alone.

Initial experiments used a cytokine maturation 'cocktail' and CpG as the positive controls (**Figure 1**)⁴⁶. Analyzing GFP(+) and GFP(-) DCs together, the Ad and Ad-LeX infected cells did not appear to become stimulated (**Figure 2**). The MFI of the co-stimulatory molecule fluorescent antibodies was not significantly different from that of the negative control. However, when the GFP(+) cells were analysed alone, these cells did up-regulate co-stimulatory molecules, indicating some stimulation. GFP(+) DCs resulting from Ad-LeX infection did not show any significant difference in levels of up-regulation compared to those infected with Ad. Intriguingly, the CpG did not cause any significant up-regulation and was not an effective positive control. The cytokine cocktail did stimulate the DCs however, as these cytokines may mask the future measurement of secreted cytokines, LPS from *Salmonella typhosa* was subsequently employed as the positive control (**Figure 3**).

The concentrations of cytokines and chemokines secreted from infected DCs were measured after 24 hours post-exposure to the viruses (**Figure 4**). No significant concentration increases over the negative control background levels were observed. Newton *et al* also reported that PBMC DCs did not produce cytokines in response to adenovirus infection⁴⁷. Both co-stimulatory molecules and cytokines were further analysed at six and 48 hours post infection and no different maturation trends were observed.

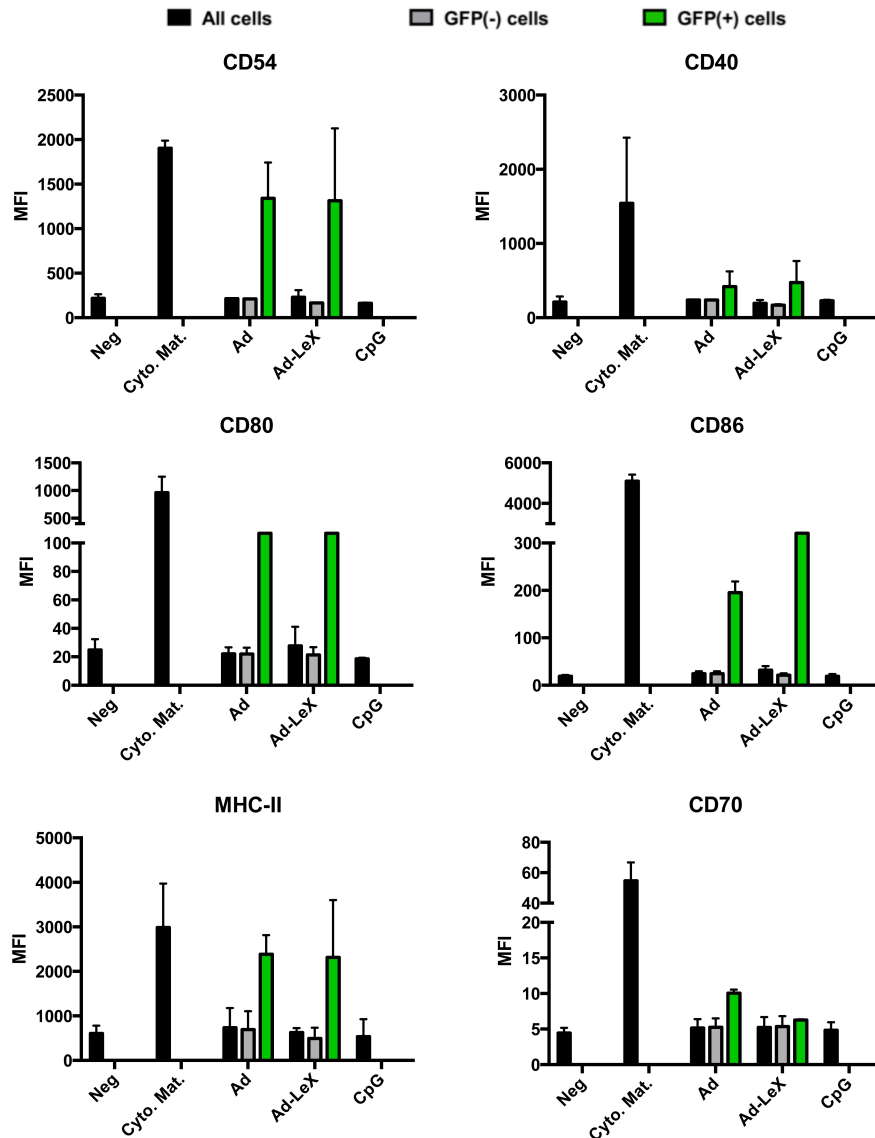


Figure 2 – Co-stimulatory molecule staining of human PBMC derived DCs, 24 hours after infection with *gfp*-Ad or *gfp*-Ad-LeX. The negative control is DCs in media only, the positive controls are cytokine-matured DCs or DCs exposed to CpG. Bars represent the average MFI of DCs from three blood donors. The error bars are standard error. Black bars represent the mean fluorescence intensity (MFI) antibody staining for the whole DC population. Grey bars are the MFI from antibody staining measured on GFP(-), non-infected DCs only, whereas green bars are GFP(+), infected cells only.

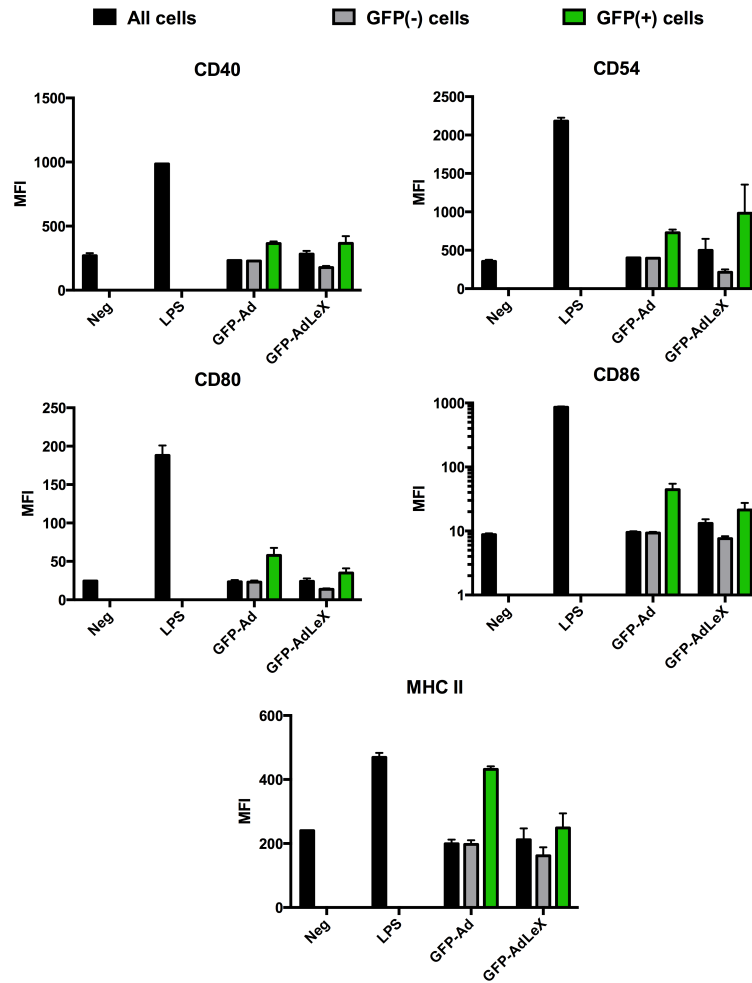


Figure 3 - Co-stimulatory molecule staining of human PBMC derived DCs 24 hours after infection with *gfp-Ad* or *gfp-Ad-LeX*. The negative control is DCs in media only, the positive control is DCs matured by LPS stimulation. Bars represent the average MFI of DCs from three blood donors. The error bars are standard error. Black bars represent the mean fluorescence intensity (MFI) antibody staining for the whole DC population. Grey bars are the MFI from antibody staining measured on GFP(-), non-infected DCs only, whereas green bars are GFP(+), infected cells only.

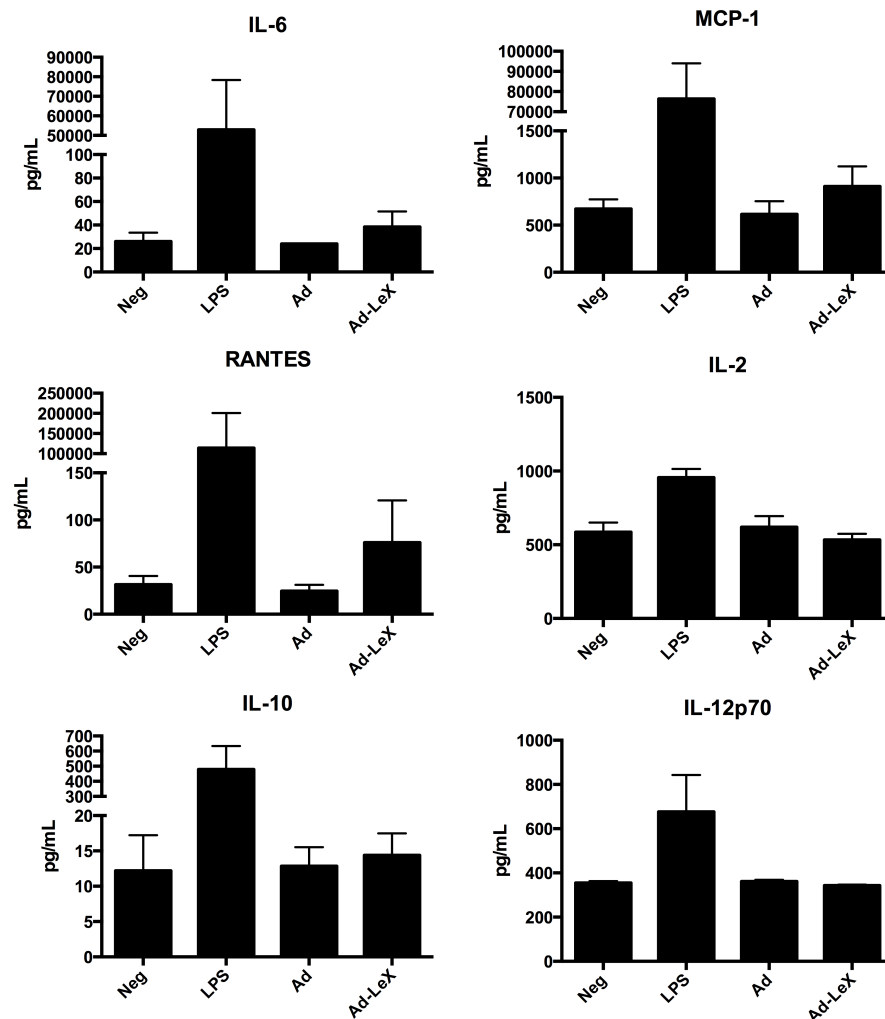


Figure 4 - Concentrations of cytokines and chemokines from supernatants taken from PBMC derived DCs 24 hours after infection with Ad or Ad-LeX. Results averaged over three blood donors and the error bars are standard error. Infection with Ad or Ad-LeX did not induce significant cytokine or chemokine secretion

DC-SIGN signaling has the potential to modify the immune response to TLR-4. Mannose-based sugars elicit DCs to excrete greater levels of IL-12 and IL-6, however fucose-based sugars (including the Lewis blood antigens Lewis X and Lewis A (LeX, LeA)) cause DCs to excrete less IL-12 and more IL-10^{10,17}. It is thus possible that the LeX-coated Ad could direct the TLR-4 response. To test this idea, DCs were infected with Ad or Ad-LeX in the presence of the LPS from *Salmonella typhosa*, a TLR-4 specific ligand. Both CD-molecule up-regulation and cytokine/chemokine concentrations were measured (**Figures 5 and 6**). The infection with Ad or

Ad-LeX did not alter the LPS induced up-regulation of CD molecules on the DCs. Nor was there any significant difference between the levels of cytokine/chemokine secretions compared to the LPS response in the absence of the viruses.

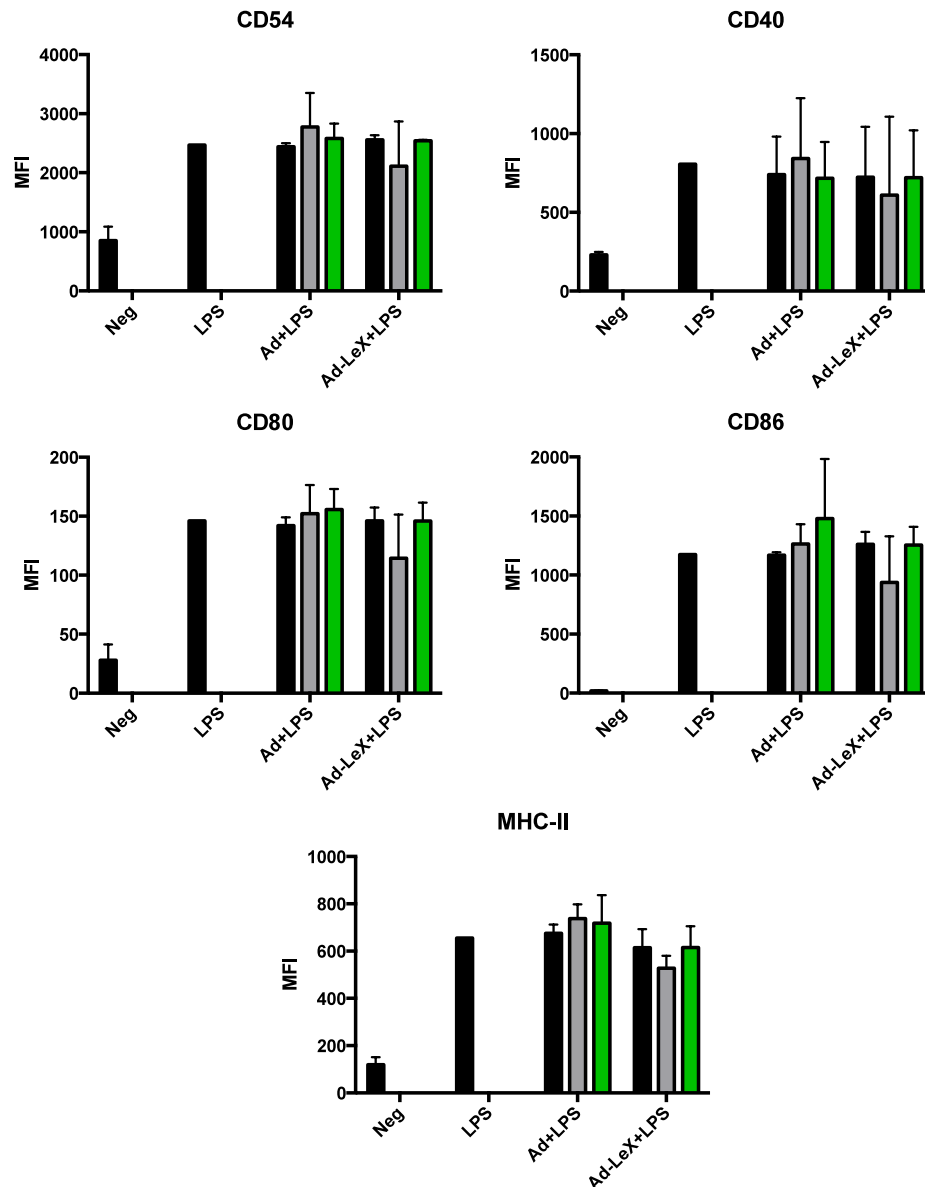


Figure 5 - Co-stimulatory molecule antibody staining of human PBMC derived DCs 24 hours after infection with *gfp*-Ad or *gfp*-Ad-LeX, in the presence of LPS. The negative control is DCs in media only, the positive control is DCs matured by LPS stimulation. Bars represent the average MFI of DCs from two blood donors, and are normalized against the LPS responses. Error bars are the standard error. Black bars represent the mean fluorescence intensity (MFI) antibody staining for the whole DC population. Grey bars are the MFI from antibody staining measured on GFP(-), non-infected DCs only, whereas green bars are GFP(+), infected cells only.

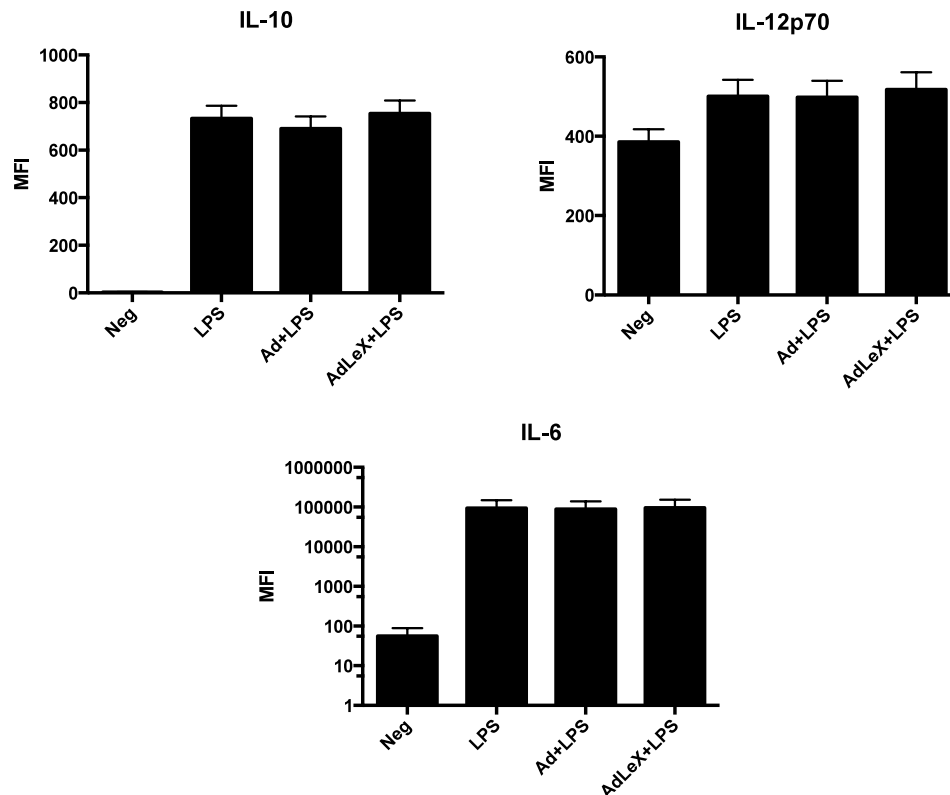


Figure 6- Concentrations of cytokines and chemokines from supernatants taken from PBMC derived DCs 24 hours after infection with Ad or Ad-LeX. Bars represent the average MFI of DCs from two blood donors. Error bars are the standard error.

5.2.2 Tg DC-SIGN(+) bone-marrow derived dendritic cell (BMDCs) stimulation

Stimulated, mature DCs infected with Ad-LeX must present the transduced antigen to T cells in order to elicit an immune response. To test this response, BMDCs from tg hDC-SIGN mice were infected with Ad-LeX and then allowed to interact with T-cells. However, the ability of the Ad-LeX to infect and stimulate these mouse BMDCs was first assessed. BMDC, extracted from mice transgenic for human DC-SIGN, were infected with Ad or Ad-LeX at 50 and 100 MOI. Blocking was achieved through a DC-SIGN blocking antibody. As observed with infections in human DCs, the *gfp*-Ad-LeX was able to more effectively transduce mouse DC compared to unmodified Ad (**Figure 7**).

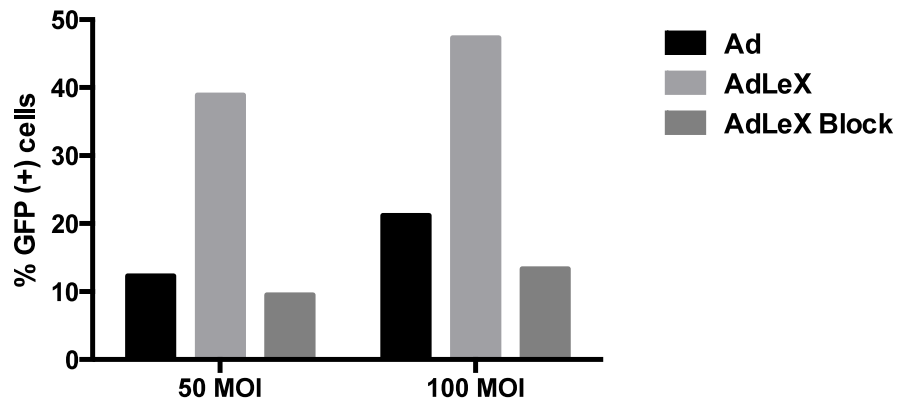


Figure 7 - The infection of transgenic mouse human DC-SIGN(+) BMDCs with *gfp*-Ad or *gfp*-Ad-LeX at 50 or 100 MOI. The DCs were incubated with the Ads for two hours, washed and GFP expression measured by flow cytometry 24 hours later. Blocking was achieved through prior incubation with anti-DC-SIGN antibody⁴⁸.

The extent of any co-stimulatory molecule up-regulation was measured using fluorescence anti-co-stimulatory antibodies and cytokine concentrations were measured by cytokine bead array (CBA) assay. CpG was used as the positive control. The BMDC were successfully stimulated, in contrast to the human PBMC derived DCs. However, the BMDCs did not up-regulate any of the tested co-stimulatory molecules in response to Ad or Ad-LeX (**Figure 8**).

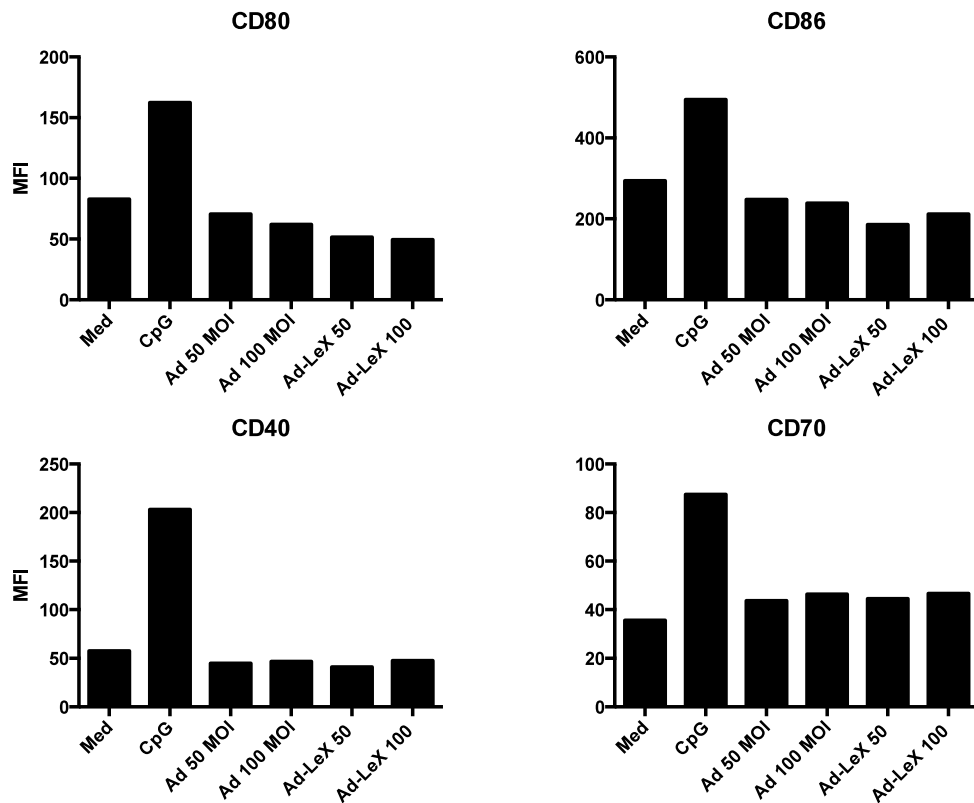


Figure 8 - Co-stimulatory molecule staining of transgenic mouse human DC-SIGN(+) BMDCs with Ad or Ad-LeX at 50 or 100 MOI. The DCs were incubated with the Ads for two hours in media, the cells were then washed into complete media and the extent of GFP expression was measured by flow cytometry 24 hours later. Blocking was achieved through prior incubation with anti-DC-SIGN antibody⁴⁸. The mean fluorescence intensity (MFI) antibody staining was recorded for the whole DC population.

The levels of TNF- α , IL-6 and IL-10 were not significantly altered from the negative control, background levels. IL-12p70 concentrations appeared to increase for infection at 50 MOI, for both Ad and Ad-LeX and for Ad-LeX with blocking antibody. At these low concentrations, the ability of the assay to resolve between the relative concentrations of IL-12p70 is questionable. This, combined with the fact the blocking antibody made no difference to the apparent Ad-LeX increase at 50 MOI, suggests that this is an anomaly. MCP-1 is a chemokine commonly secreted in response to viral infections⁴⁹. MCP-1 secretion was increased in response to Ad and Ad-LeX infection, proportional to MOI. Ad-LeX infection induced significantly

greater MCP-1 than Ad at the same MOI, suggesting that the DCs are stimulated by the Ad-LeX. DC-SIGN blocking reduced the Ad-LeX dependent secretion of MCP-1 (Figure 9).

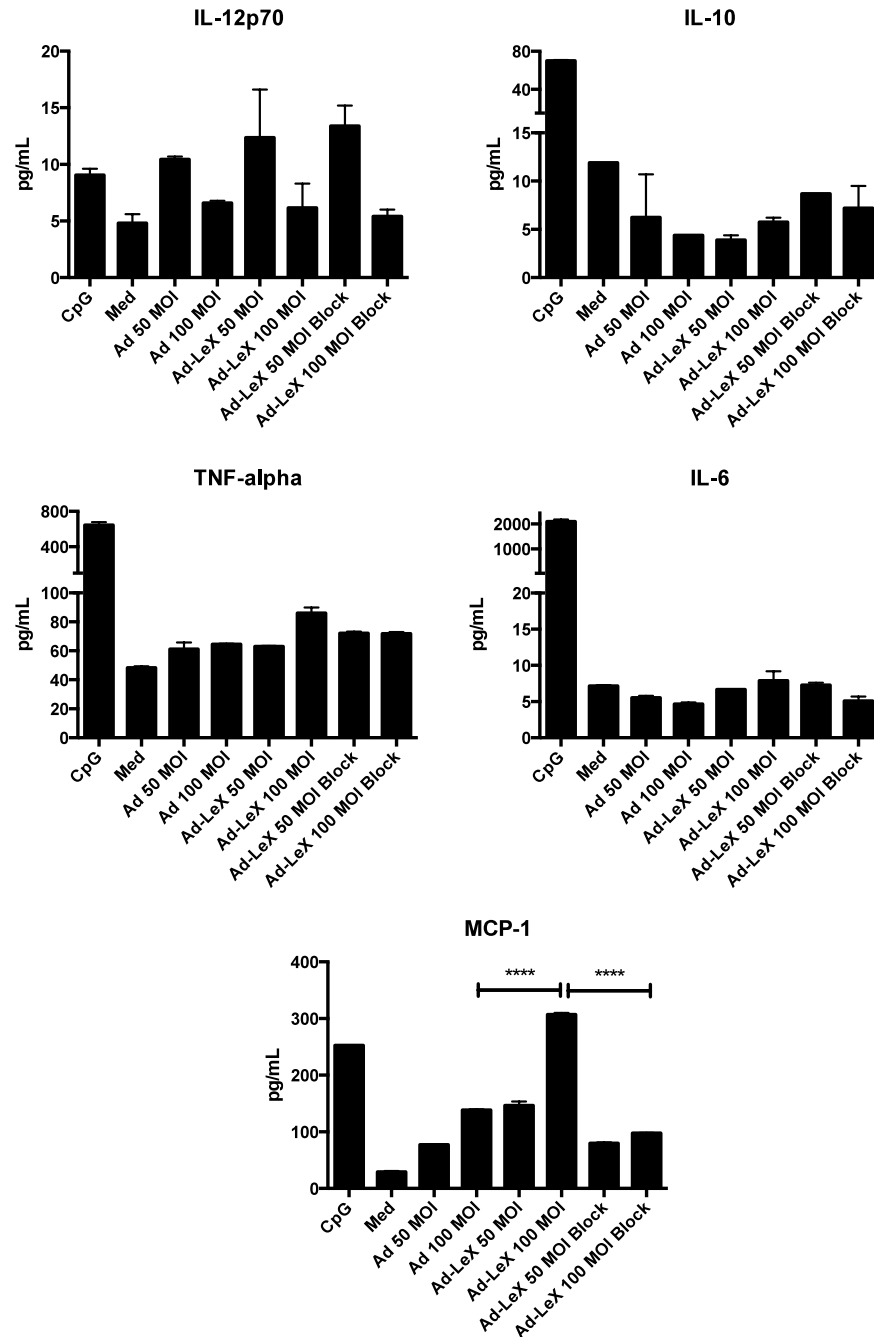


Figure 9 - Concentrations of cytokines and chemokines from supernatants taken from transgenic mouse human DC-SIGN(+) BMDCs 24 hours after infection with Ad or Ad-LeX at 50 or 100 MOI. Blocking was achieved through prior incubation with anti-DC-SIGN antibody⁴⁸. Bars represent the average of technical triplicates and the

error bars are standard error. . Statistics performed were an unpaired T-test where **** $p < 0.001$.

5.2.3 T cell proliferation resulting from Tg DC-SIGN(+) bone-marrow derived dendritic cell (BMDCs) infection.

Transgenic human DC-SIGN mouse BMDCs were infected with Ad or Ad-LeX encoding for OVA protein (*ova*-Ad, *ova*-Ad-LeX) at 100, 250 and 500 MOI. Ad-LeX infected the DCs more effectively than the Ad, GFP transduction was proportional to MOI (**Figure 10a**). BMDCs transduced with *ova*-Ad-LeX were incubated with T cells isolated from either OT-I or OT-II mice, and T cell proliferation measured after 3 and 5 days by both ^3H -thymidine incorporation (**Figure 10b** and **Figure E3 for controls**) and internal CFSE staining (**Figure 11** and **Figure 12**). OT-I and OT-II T cells express T cell receptors (TCRs) specific for the MHC-I or MHC-II restricted peptides of the OVA protein. Both assays showed a large relative increase in the expansion of the OT-I T cell population in response to BMDCs infected with *ova*-Ad-LeX. Notably, ^3H -thymidine incorporation demonstrated a 10- fold greater proliferation, induced by Ad-LeX infected BMDCs, compared to those injected with unmodified *ova*-Ad, or Ad expressing an irrelevant protein (*gfp*-Ad-LeX). Increased OT-II T cell proliferation was also observed, although to a lesser extent. This could be an indication of successful cross presentation of OVA. The addition of blocking anti-DC-SIGN antibody during transduction prevented subsequent OT-I T cell proliferation. Raf-1 is part of the DC-SIGN signalosome¹⁷. Raf-1 inhibition did not affect the extent of the OT-I T cell proliferation indicating that DC-SIGN did not play role in the antigen presentation process.

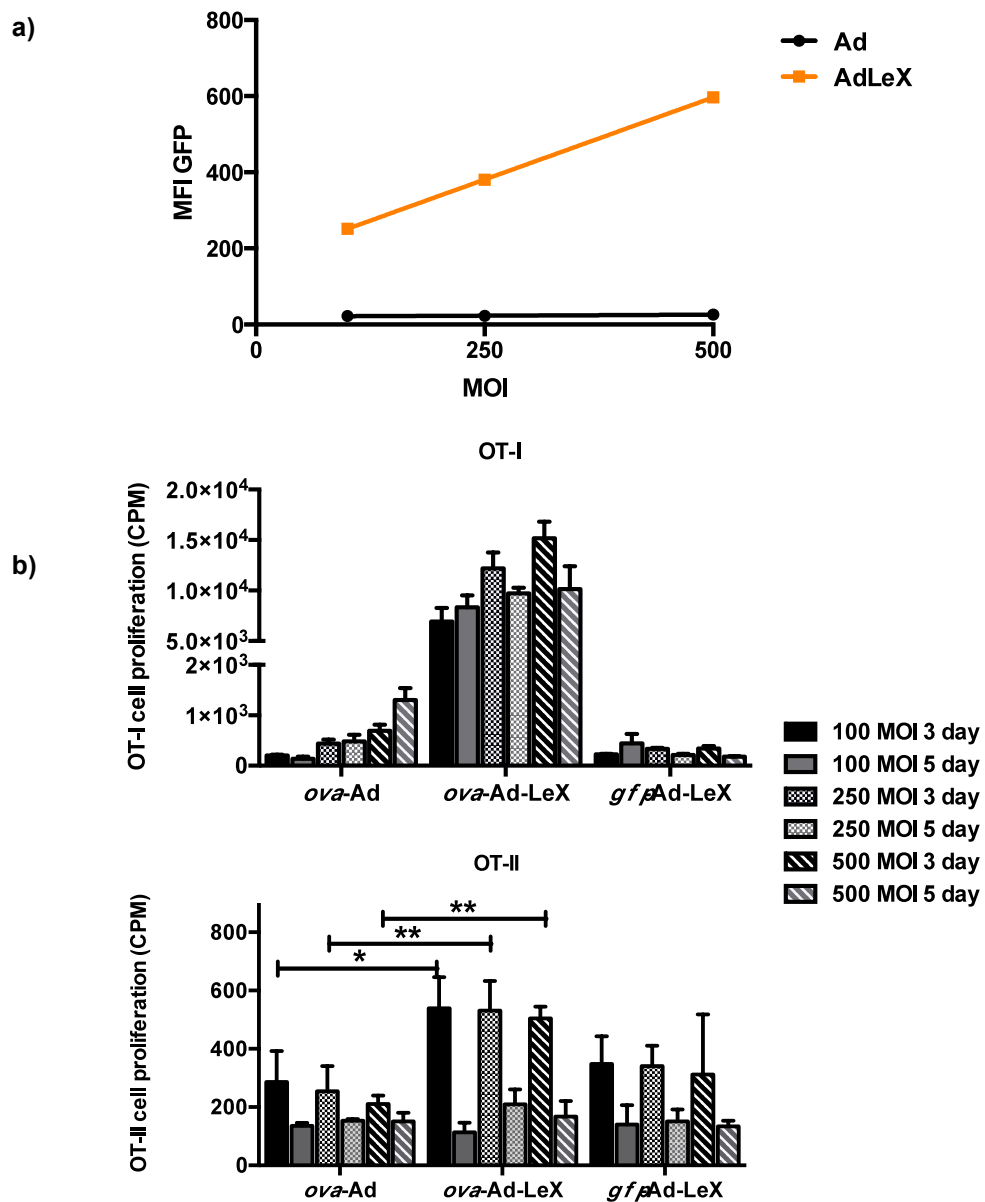


Figure 10 – a) The infection of transgenic mouse human DC-SIGN(+) BMDCs with *gfp*-Ad or *gfp*-Ad-LeX at 100, 250 or 500 MOI. The DCs were incubated with the Ads for two hours, the cells were then washed and the extent of GFP expression was measured by flow cytometry 24 hours later. T cell proliferation of b) OT-I and c) OT-II mouse T cells after incubation with mouse BMDCs infected with *ova*-Ad-LeX or *ova*-Ad. *gfp*-Ad-LeX was included as a non-relevant protein control. The extent of proliferation was measured by ^3H -thymidine incorporation over a 16h period at 3 and 5 days post-BMDC incubation. Bars represent the mean of technical triplicate and the error bars are the standard error. Statistics performed are 2-way ANOVA followed by Bonferroni's multiple comparison test where * p < 0.05; ** p < 0.01; **** p < 0.001.

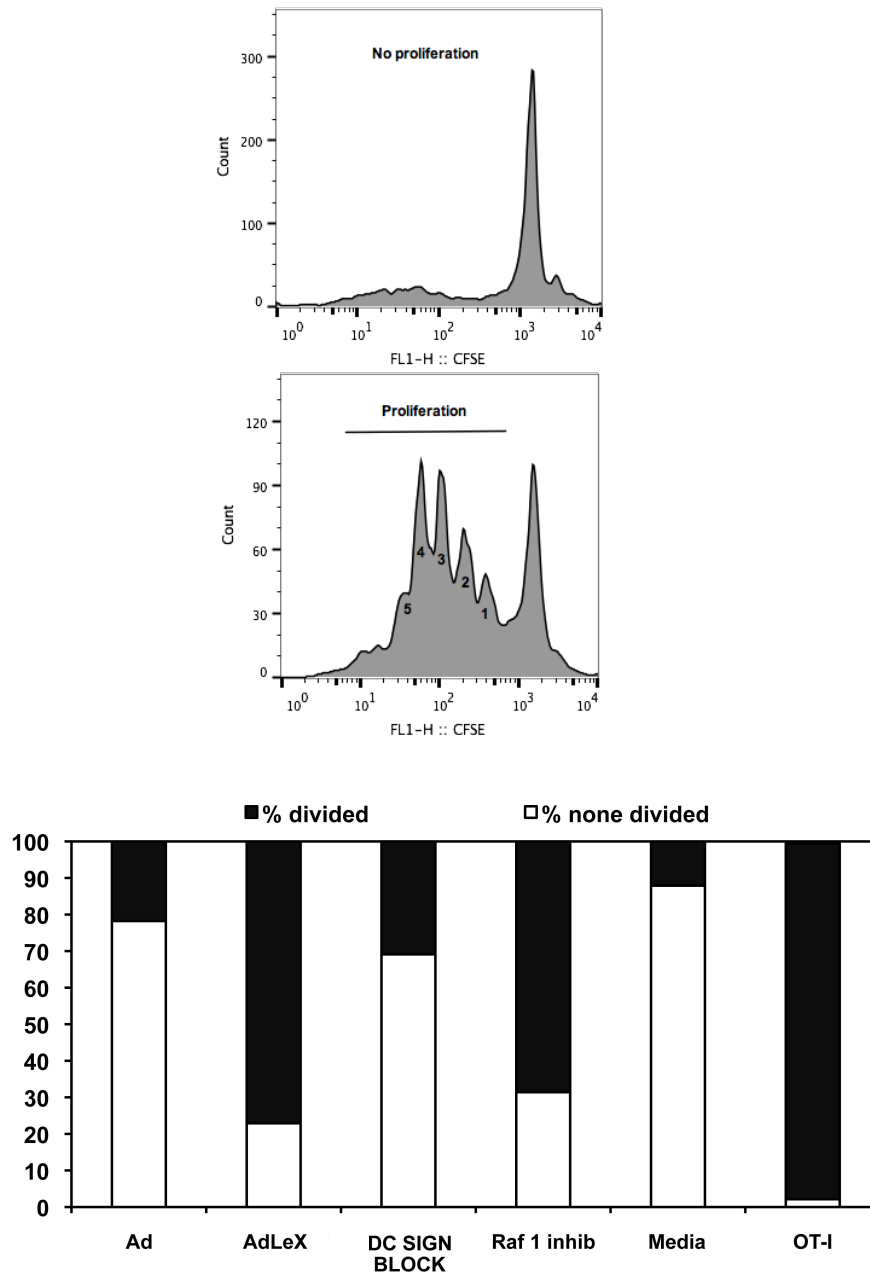


Figure 11 -) T cell proliferation of OT-I mouse T cells after incubation with Tg hDC-SIGN mouse BMDCs infected with ova-Ad or ova-Ad-LeX at 100 MOI. Proliferation was measured by the decrease in the internal stain CFSE upon T cell division. The histogram of CFSE intensity shows an example where no T cells have divided and an example where T cells have divided up to 5 times. The % of divided cells was recorded as those cells that had divided one or more times. BMDCs infected with Ad-LeX induced greater levels of OT-I T cell proliferation. This increased response was abrogated upon the blocking of DC-SIGN during the initial DC infections. Raf-1 blocking did not effect the proliferation.

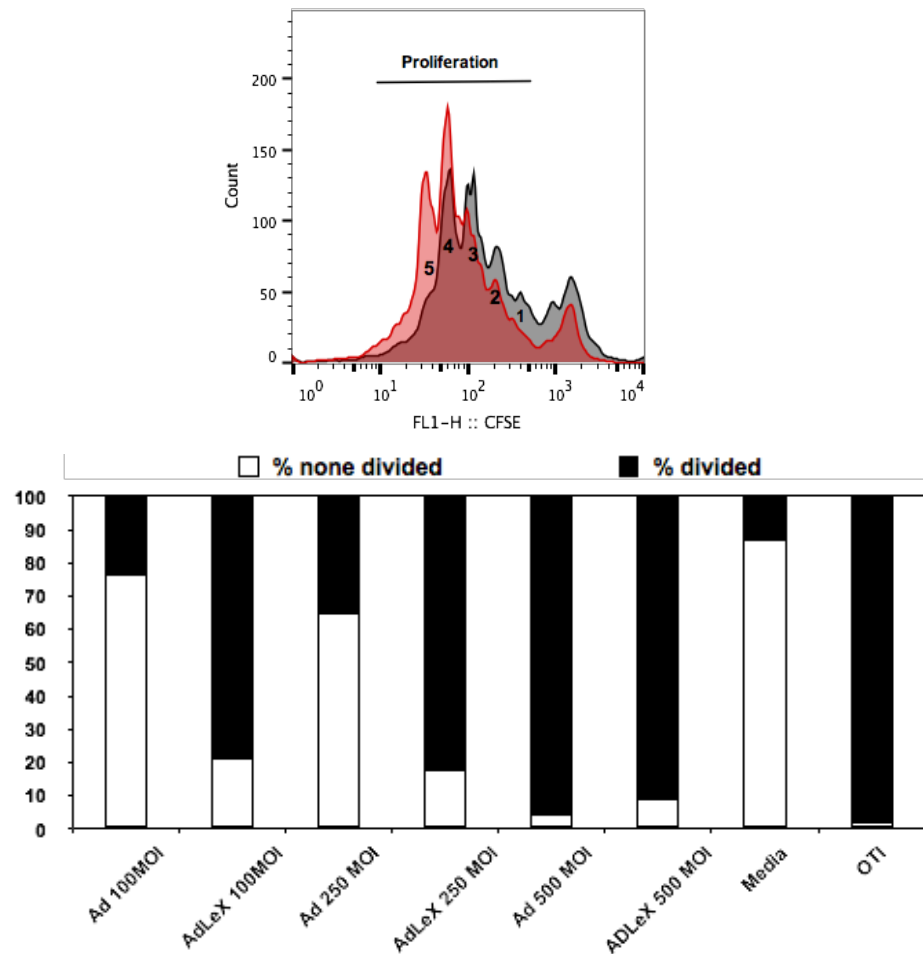


Figure 12 -) T cell proliferation of OT-I mouse T cells after incubation with Tg hDC-SIGN mouse BMDCs infected with ova-Ad or ova-Ad-LeX at 100, 250 and 500 MOI. The % of divided cells was recorded as those cells that had divided one or more times Proliferation was measured by the decrease in the internal stain CFSE upon T cell division. BMDCs infected with Ad-LeX induced greater levels of OT-I T cell proliferation at 100 and 250 MOI. At 500 MOI Ad and Ad-LeX the same percentage of cells had proliferated however the extent of the proliferation was greater arising from Ad-LeX. The histogram of the CFSE intensity shows that higher numbers of T cell splitting was achieved from BMDCs infected with Ad-LeX (Red) over BMDCs infected with Ad (grey).

5.3 Discussion

Collectively, these results demonstrate that the modification of *ova*-Ad with LeX enhances antigen presentation of OVA protein and subsequent anti-

OVA T cell proliferation, particularly CD8 T cells. CD4 T cell proliferation was also significantly increased, although to a lesser extent.

The Ad-LeX did not appear to modulate the immune TLR-4 response. If the Ad-LeX did show some immune modulation effect this could alter the immune response against the transgene and have a negative impact on the effectiveness of Ad-LeX as a DNA vaccine vector. The inability of Ad-LeX to modulate the DC immune response could indicate that the virus is not internalizing via DC-SIGN. Instead, in a similar manner to how Ad uses CAR, the Ad-LeX could be anchoring with DC-SIGN but still internalizing via the integrin pathway. Such an internalization pathway would not stimulate previously reported DC-SIGN immune modulation mechanisms. Future experiments could test this theory by blocking integrin binding, through the presence of RDG peptides for example, and measuring any inhibition of Ad or Ad-LeX infectivity. Studies have investigated immune modulation via DC-SIGN with artificially fucosylated gold nanoparticles and found no effect⁵⁰. It appears that the controlling factors initiating DC-SIGN induced modulation are more complex than LeX binding alone. It is plausible that further factors, such as the extent of DC-SIGN clustering and multivalent binding, may also play a role.

5.4 Experimental

Transfection of dendritic cells. Immature DCs derived human PBMC were infected with *gfp*-Ad or *gfp*-Ad-LeX at 1000 MOI. The viruses were diluted in PBS to a final volume of 10uL and were then added to the cells. DCs were matured for positive controls by either cytokine cocktail for a total of 48hrs (PGE2 (1μg/mL), IL1β (10μg/mL), IFNγ (20μg/mL), TNFα (50μg/mL), additional to the existing IL4 or GM-CSF, or by incubation with LPS from *Salmonella typhosa* (0.1 ug/mL) (Sigma). After 24 hours, supernatants were removed and the cells were stained for maturation markers. Luminex beads were used for the measurement of secreted cytokine concentrations (RnD systems custom kit). The cells were suspended in 500μL and split across 4

wells in a V bottomed 96 well plate. The plate was centrifuged (1400rpm, 3 min, 4°C) and the cells were washed with 100µL of cold buffer (PBS + 2% BSA), kept on ice. To the cells, 50µL of the relevant antibody dilutions were added (BioLegend, APC conjugated). The antibodies were left on the cells for 30 min on ice before being washed off with buffer. The cells were then finally suspended in 200µL PBS before being taken for flow cytometry analysis.

Breeding of Tg hDC-SIGN mice. Two breeding males of C57BL/6, transgenic for human DC-SIGN, were gifted to us by the Sparwasser lab ⁴³ and bred in our facilities. The line was maintained by breeding heterozygous with heterozygous mouse, the homozygous mice are infertile (identified by grey coloured fur). Heterozygous mice between 6-14 weeks were used for experiments.

Tg hDC-SIGN mouse PCR. To each ear clip 300µL of lysis buffer (100mM Tris-HCl pH 8.5, 10mM EDTA, 0.2% SDS and 200mM NaCl) was added followed by 3µL of proteinase K (20mg/mL in H₂O) and the mixture left to incubate at 55°C overnight. The sample was then centrifuged (13,000xg, 5min) and the supernatant removed. To the supernatant 300µL isopropanol was added, vortexed and then centrifuged. The isopropanol was poured off and replaced with 1mL of 70% ethanol, centrifuged and again the alcohol was poured off. The sample was left to dry completely before being dissolved in 100µL of water. The DNA concentration was measured using UV measurement at 260nm and was found to vary between samples. The PCR reaction mixture was as follows: 5µL of standard buffer, 2.5µL of each CD11c and DC-SIGN primers, 1µL of DNA extracted, 0.5µL dNTP, 0.125µL OneTaq® (NEBiolabs) and 8.375µL of MilliQ water. The primer sequences were:

CD11c: GAGACTCTGGCTACTCATCC and CCTTCAGCA
AGAGCTGGGGAC,

DC-SIGN: CGGGATCCGAGTGGGGTGACATGAGTGACT and
ACGCGTCGACAAAAGGGGGTGAAGTTCTGCTACG.

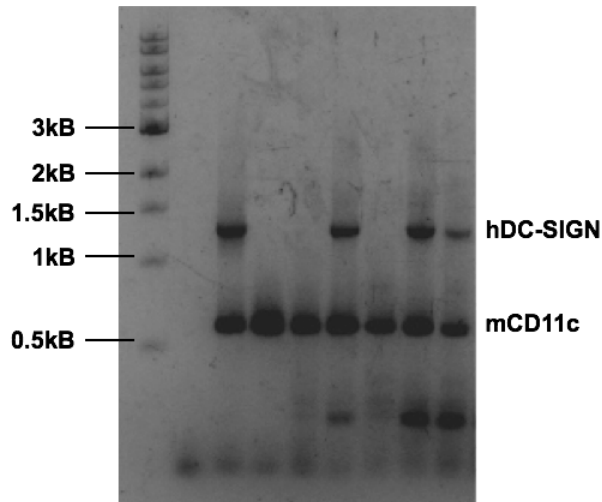


Figure E1- The PCR results of mice 1-18. Those displaying a band between 1000-1500 bps are mice (+) for hDC-SIGN.

Temperature	Time	Cycles
95°C	00:30 min	
94°C	00:30 min	
59°C	00:30 min	35x
72°C	1:00 min	
72°C 15°C	10 min ∞	

Immature BMDCs were differentiated from transgenic human DC SIGN mice (Tg-hDCSIGN). Bone marrow from the femurs and tibiae of tg-hDC-SIGN mice was extracted by flushing the bones with PBS. The cells were then seeded (2×10^6) in bacteriological Petri dishes (100mm) in 10mL of IMDMc media (IMDM, 10% inactivated FBS, P/S 1:1000, glutamax 1:1000 and β metcaptoethanol 50 μ M) containing 20ng/mL rmGM-CFS (Peprotech). After 3 days, another 10mL of IMDMc containing 20ng/mL rmGM-CFS was added. At days 6 and 8, half of the culture supernatant was collected,

centrifuged, and the cell pellet re-suspended in 10 ml fresh IMDMc containing 20ng/mL of rmGM-CSF, and given back into the original plate. The cells were then ready to use⁵¹.

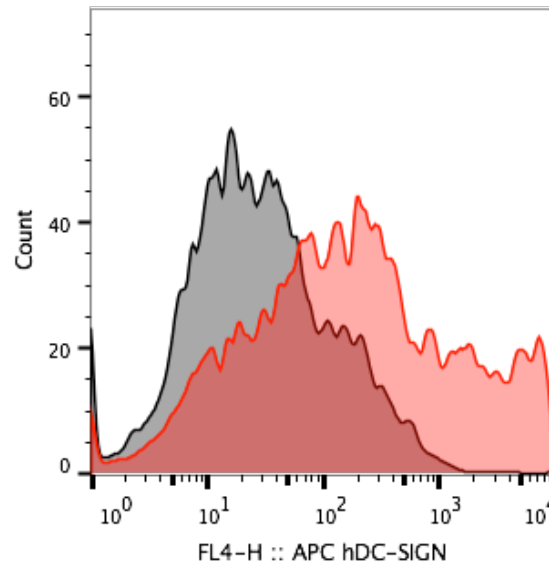


Figure E2- Fluorescence antibody staining of tg hDC-SIGN(+) (red) or hDC-SIGN(-) (grey) mouse BMDCs with anti-human-DC-SIGN APC antibody. DCs from DC-SIGN(+) mice bound more antibody.

OT-I and OT-II T cell extraction. Spleen and lymph nodes were extracted from the OT-I or OT-II transgenic mice, the organs were then pushed through a wire mesh and washed with 10% IMDMc to a volume of around 8mL. The resulting cell suspensions were then centrifuged (1400rpm, 10 min, 4°C) and the media removed. The pellet was broken up and 2.5mL of ACK lysis buffer was added to the pellet on ice. This was left for 1 minute before 10mL of 10% IMDMc was added and the cells quickly spun again (1400rpm, 10 min, 4°C). The pellet was re-suspended in PBS 1% BSA and a count was taken. The kit Dynabeads Untouched Mouse CD8(+)/CD4(+) cell by Invitrogen was used to extract the CD8+ and CD4+ T cells respectively. An additional positive selection step was then used to select only the CD62L(+) CD4(+) T cells. (CD62L antibody coated beads from the MACs CD4(+) CD62L(+) T cell Isolation Kit II Mouse was used). These T cells we

then added to the previously primed BMDCs at 5×10^4 cells/well/50 μ L in 10% IMDMc media.

T cell proliferation. Immature BMDCs were differentiated from transgenic human DC SIGN mice (tg-hDCSIGN). The cells were re-suspended in IMDMc media (IMDM, 10% or 2% heat inactivated FBS, P/S 1:1000, glutamax 1:1000 and β metcaptoethanol 50 μ M). The cells were then plated on U-bottomed 96 well plates 2.5×10^4 cells/well/50 μ L. To these cells *ova*-Ad-LeX or *ova*-Ad dilutions were added in 50 μ L of the 2% IMDMc at MOI 100, 250 and 500. OVA protein was added to control BMDCs at a final concentration of 500 μ g/mL followed by 2 fold serial dilutions. OT-I specific peptide OVA₂₅₇₋₂₆₄ was added to CD8(+) T cells as a further positive control starting at 1 μ g/mL, final concentration, and followed by 5 fold serial dilutions. OT-II peptide OVA₃₂₃₋₃₃₉ was added, starting at 10 μ g/mL followed by 5 fold serial dilutions. The virus and proteins were left on the BMDCs for 2 hours at 37°C. 100 μ L of media was then added to the cells and the plates were centrifuged (1200 rpm, 3 min, at RT low brake). This washing process was repeated 3 times. The cells were finally suspended in 50 μ L of 10% IMDMc. After 2 hours incubation with the infected BMDCs, the cells were extensively washed and either 5×10^4 CD4(+) T cells enriched from OT-II tg mice⁵² or CD8(+) T cells from OT-I transgenic mice⁵³ were added. The plates were then left at 37°C for either 3 or 5 days before ³H-thymidine was added to the wells and left for 16 hours. Cells were harvested onto filters and ³H-thymidine incorporation was assessed using a beta counter.

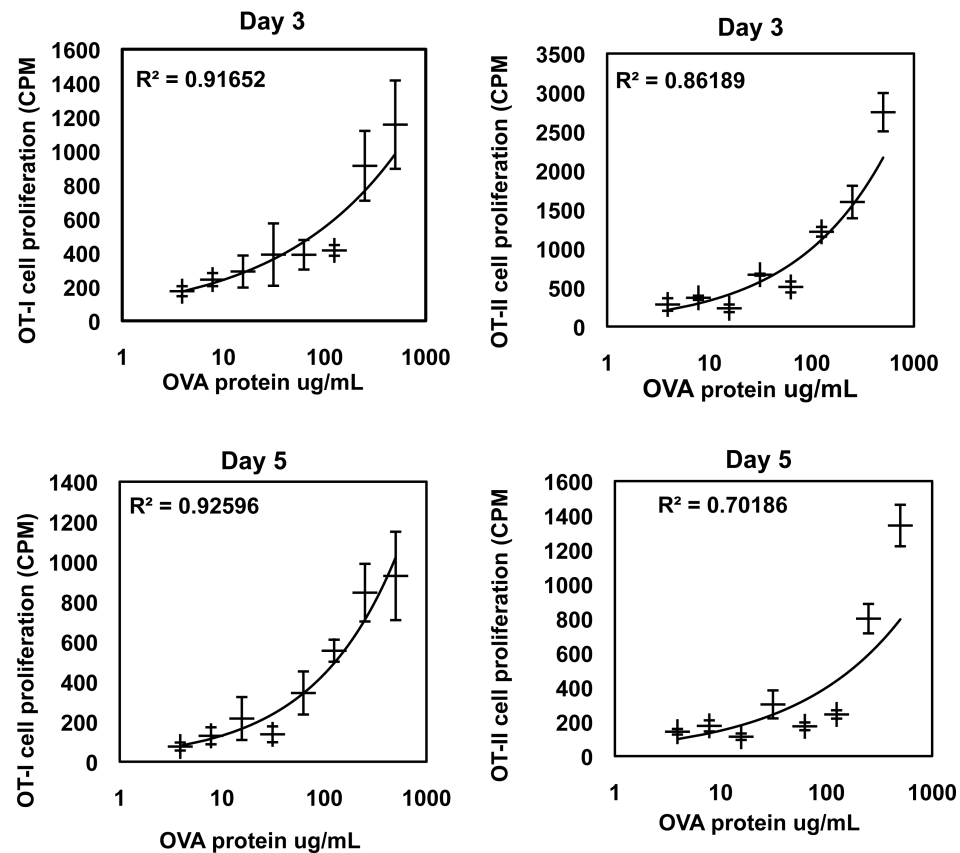


Figure E3 – The OVA controls for the T cell proliferation assay by ³H-thymidine incorporation

CFSE T cell proliferation assay. BMDCs were differentiated from transgenic human DC SIGN mice (tg-hDCSIGN) and plated in two 96 well plate 2.5×10^4 c/well/25 μ L in 2% IDMDc. The cells were then incubated for 45min at 37°C with either 25 μ L of anti DC SIGN blocking antibody (20 μ g/mL in final concentration in 100 μ L), Raf-1 inhibitor (20 μ M final concentration in 100 μ L) or media. Next, *ova*-Ad or *ova*-Ad-LeX was added to the BMDCs in 50 μ L of 2% IDMDc at 100, 250 or 500 MOI. *ova*-Ad was only added to BMDCs which had not been blocked. Positive controls of either OTI- (1 μ L/mL final concentration in 100 μ L) or OT-II (10 μ L/mL final concentration in 100 μ L) peptides were also included without any prior blocking. The virus and peptides were incubated with the cells for 2 hours at 37°C. The BMDCs were then washed 3 times with media (1200 rpm, 3

mins, low brake) and finally suspended in 50 μ L 10% IMDMc. OT-I and OT-II T cells were permeabilised using cell permeabilisation buffer (eBioscience) then incubated with a final concentration of 1 μ M of carboxyfluorescein succinimidyl ester (CFSC) stain before being added to the previously primed BMDCs at 5 $\times$ 10⁴/well/100 μ L. The cells were left to incubate together for 3 days at 37°C. Finally, the cells were transferred to a V-bottomed well plate before being washed 3 times with buffer (PBS, 2% BSA). The OT-I plate was then stained with anti CD8(+) APC-antibody and the OT-II plate with CD4(+) for 20mins at 4°C. The samples were then washed and analyzed by flow cytometry. T cells were gated for either CD4 or CD8 and then the extent of proliferation was assessed by the percentage of populations showing a reduction in CFSE intensity.

Cytokines and Co-stimulatory molecules. BMDCs were differentiated from transgenic human DC SIGN mice (tg-hDCSIGN) and plated in a 24 well plate at 5 $\times$ 10⁵ cells/well/200 μ L in 2% IMDMc. To the cells either 100, 250 or 500 MOI of *gfp*-Ad-LeX or 500 MOI of *gfp*-Ad were added in 50 μ L of the media. CpG was used as a positive control at a final concentration of 0.5 μ g/mL. The virus was incubated with the cells for 2 hours at 37°C. The cells were then diluted in 500 μ L of media before being spun (1200 rpm, 3 min, 4°C). 600 μ L of media was then removed from the wells and the cells were washed with a further 800 μ L of media and centrifuged. This process was repeated a total of 3 times before the cells were finally suspended in 1mL of 10% IMDMc. The BMDCs were then left for 6 hours or 24 hours at 37°C. The supernatant was then removed and stored at -80°C for later analysis. The cells were suspended in 500 μ L and split across 4 wells in a V bottomed 96 well plate. This plate was spun (1400rpm, 3 min, 4°C) and the cells were washed with 100 μ L of buffer (PBS, 2% BSA), kept on ice. To the cells, 50 μ L of the relevant antibody dilutions were added. The antibodies were left on the cells for 30 min on ice before being washed off with buffer. The cells were then finally suspended in 70 μ L of the buffer before being

taken for flow cytometry analysis.

Cytometric Bead Array (CBA) assay. The supernatants of BMDC samples 24 hours post infection with *gfp*-Ad and *gfp*-Ad-LeX, and controls (see above), were stained with cytokine antibody coated fluorescent beads, BD Cytometric Bead Array . 10 μ L of media sample were combined with 10 μ L of bead mixture. A serial dilution of cytokine standards was prepared from the kit, these were also stained with the beads. The beads were left to bind for 2 hours at RT covered from light. The beads were then centrifuged and washed with the buffer from the kit. The samples were then analyzed by flow cytometry and the results processed using the CBA staining program on CellQuest.

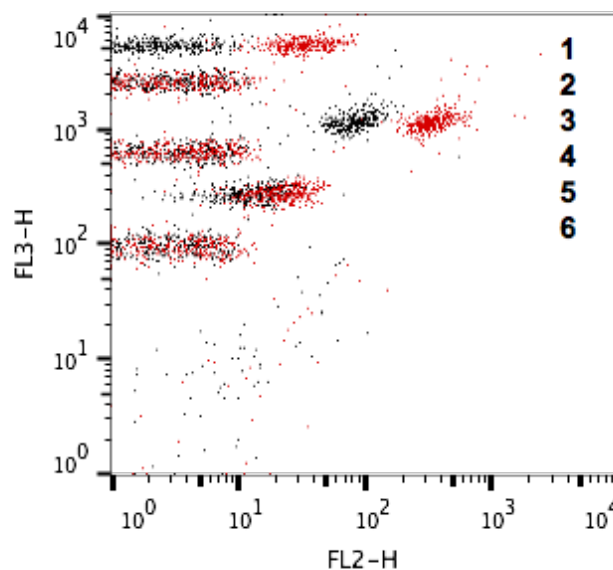


Figure E4- An example of the flow cytometry analysis of the cytometric Bead Array (CBA) assay. Each group on the FL-3 axis (1-6) represents a particular bead bound to its specific analyte. The extent of the binding to the bead is then detected by subsequent anti-analyte antibody binding (FL2 channel). This intensity of fluorescence is then compared to a standard curve.

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6. *In vivo* vaccination of tg hDC-SIGN mice

6.1 Results

6.1.1 *In vivo* vaccination studies

To further assess the ability of the synthetic glycovirus Ad-LeX to induce both cellular and humoral adaptive immunity, *in vivo* vaccination experiments were conducted. Female heterozygous hDC-SIGN transgenic (tg) mice were immunized with a mixture of both unmodified *ova*-Ad and luciferase expressing Ad (*luc*-Ad), or *ova*-Ad-LeX and *Luc*-Ad-LeX, with identical boosts at 2 weeks.

Tail bleeds were taken every 2 weeks for 8 weeks and anti-OVA and anti-Ad antibodies titres were assayed (**Figure 1a and b**). No significant differences were observed in the anti-OVA antibody titres between Ad and Ad-LeX in either the hDC-SIGN tg mice or the DC-SIGN(-) littermates, consistent with the low OT-II proliferation seen *in vitro*. The anti-Ad antibody titre was lower from mice vaccinated with Ad-LeX compared to naked Ad; consistent both with the blocking of known epitopes in the Ad and the typically poor ability of glycoconjugates to elicit robust immunogenic responses¹. Sera from tg hDC-SIGN(+) mice alone was also analysed for Ad-LeX antibody titres (**Figure 1c**). Sera from mice vaccinated with Ad did bind the Ad-LeX capsid to a similar extent as to the Ad capsid. Equally, sera generated from vaccination with Ad-LeX cross-reacted with the Ad capsid. These results indicate that sera from mice vaccinated with the Ad contained greater levels of antibody against both Ad and Ad-LeX capsids, compared with sera from Ad-LeX vaccination. This could be due to DC-SIGN targeting enhancing anti-Ad antibody production. However, this is not a statistically significant result and would need to be repeated at higher group numbers.

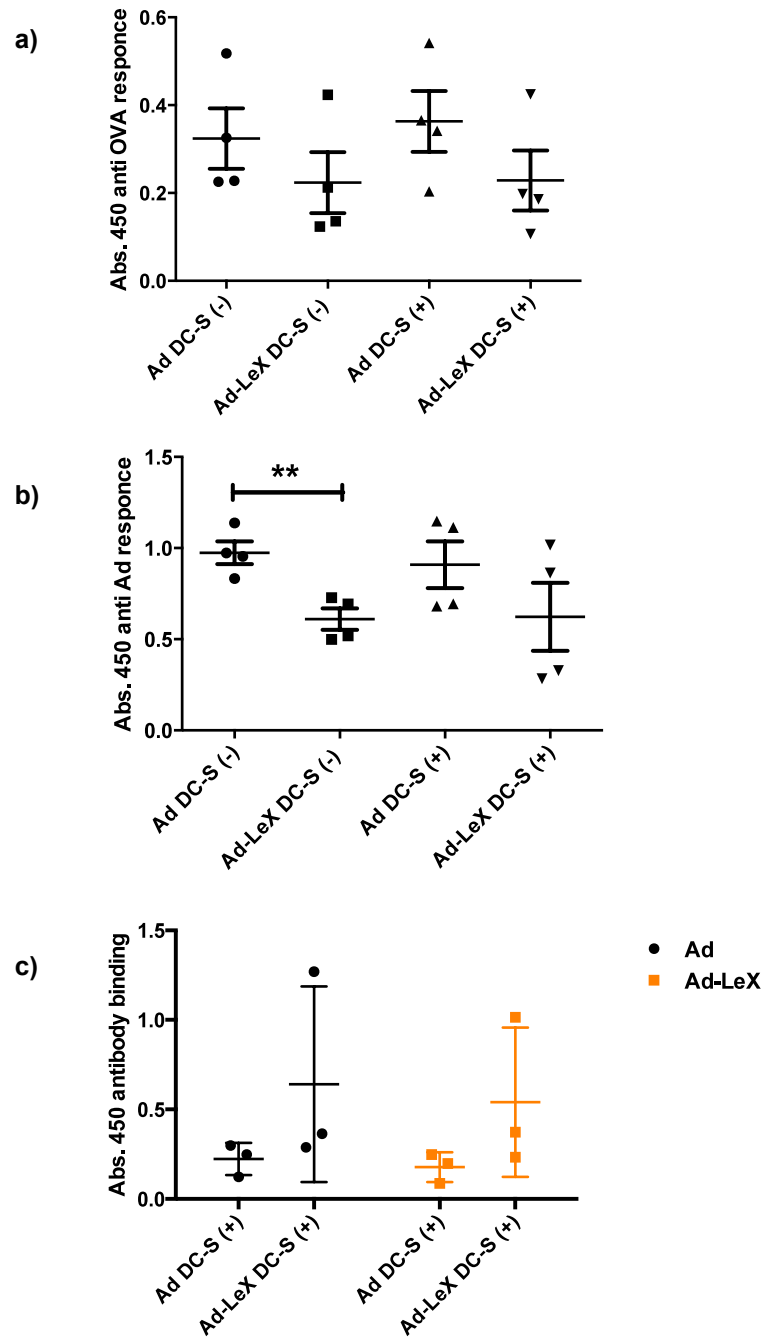


Figure- 1. Antibody reactions from sera of mice two weeks after a prime and boost vaccination with either ova-Ad or ova-Ad-LeX. ELISA plates were coated with a) OVA protein, b and c) whole Ad or c) Ad/Ad-LeX. No significant difference between the anti-OVA response was observed between the Ad and Ad-LeX vaccination, however the Ad-LeX did produce fewer anti Ad antibodies. Sera from a separate vaccination experiment were used to test the cross reactivity against Ad and Ad-LeX. Statistics performed are 2-way ANOVA followed by Bonferroni's multiple comparison test where * $p < 0.05$; ** $p < 0.01$; ** $p < 0.001$.**

The number of anti-OVA CD8 T-cells generated from vaccination with *ova*-Ad and *ova*-Ad-LeX was measured using a fluorescent pentamer displaying the immunodominant MHC-I peptide, OVA₂₅₇₋₂₆₄. 12 days after injection with *ova*-Ad or *ova*-Ad-LeX, the spleens and proximal inguinal lymph nodes of the mice were extracted and homogenised. The cells were stained with OVA peptide-bearing pentamer, anti-CD8 and CD3 antibodies. The percentage of the CD8 T-cell population pentamer (+) could then be measured by flow cytometry (**Figure 2**). CD3(+) CD8(-) population (CD4 T-cells) did not show any reactivity towards the pentamer, as expected. Cell populations extracted from the spleens of tg DC-SIGN(+) mice vaccinated with *ova*-Ad and *ova*-Ad-LeX contained the same percentage of pentamer-reactive CD8 T-cells, 5% of the total CD8. Cells extracted from the proximal inguinal lymph nodes of the mice did not show any reactivity towards the pentamer. It was concluded that this lymph node may not be the primary draining lymph node for the injection site (back leg muscle), further investigations were conducted to identify the true draining lymph node (See DC *in vivo* tracking).

These initial injections of virus contained 1.5×10^9 virus particles (vps) of *ova*-Ad. It was possible that the amount of virus might be saturating the production of T-cells. The literature suggests a maximum number of T-cells produced against Ad injections at high concentrations (at around 5-8%²⁻⁶). Therefore, it was hypothesised that at lower virus concentrations any difference between Ad and Ad-LeX might be observed. A titration of three decreasing concentrations of a *luc*-Ad/*ova*-Ad 1:1 mixture were injected into mice (n=2). After 24 hours, luciferin expression was measured by bioluminescence (**Figure 3**). The images demonstrate the expected decrease in luciferase expression with decreasing virus concentrations. After 12 days, the spleens of the mice were extracted, homogenised and the cells were stained with OVA peptide bearing pentamer, and anti-CD8 and CD3 antibodies (**Figure 3**). It was observed that the percentage of pentamer(+) CD8 T-cells did not decrease until the virus concentration was 1.5×10^7 vp/injection of *ova*-Ad. A repeat experiment, comparing the anti-OVA T-

cell numbers from *ova*-Ad or *ova*-Ad-LeX, was then conducted at 1.5×10^7 vp/injection. Tg hDC-SIGN(+) mice and DC-SIGN(-) littermate controls were vaccinated (**Figure 4**). Similar to vaccinations at higher virus concentrations, no differences in the percentage of pentamer reactive CD8 T-cells were observed. It is possible that at higher group numbers some differences might be observed.

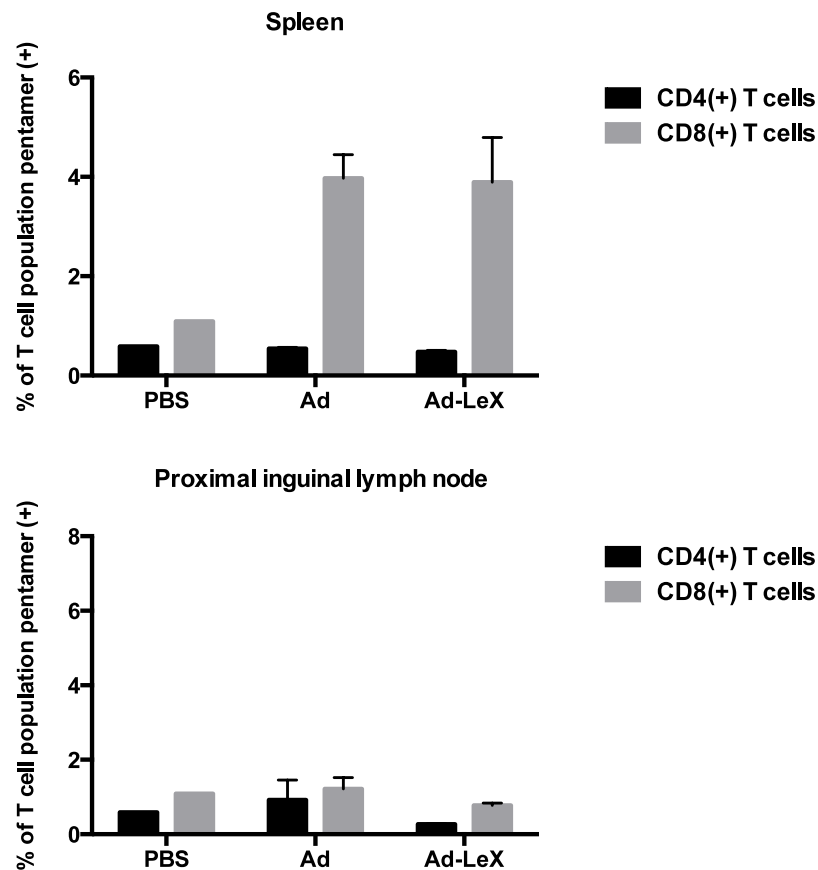


Figure 2- Tg hDC-SIGN(+) mice were injected with 1.5×10^9 vp of *ova*-Ad or *ova*-Ad-LeX. After 12 days the mice were sacrificed and splenocytes harvested. The cells were then stained for CD8 and for anti OVA specificity using an OVA peptide-bearing fluorescent pentamer. There was no significant difference between the percentages of OVA reactive CD8 T-cells from mice injected with Ad or Ad-LeX. Bars represent the average of present biological repeats (n=4) and the errors bars are the standard error.

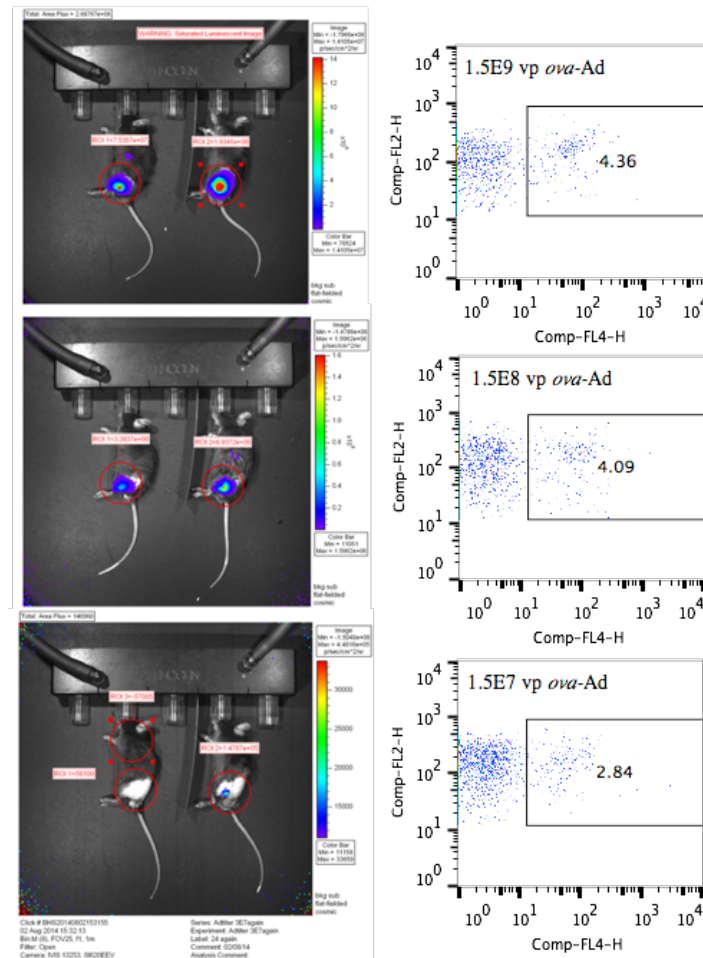


Figure 3 - Mice were injected with decreasing concentrations of a 1:1 mixture of *luc* and *ova*-Ad. After 24 hours, the mice were imaged for bioluminescence and on day 12 the mice were sacrificed and splenocytes harvested. The cells were then stained for CD8 and for anti OVA specificity, using an OVA peptide-bearing fluorescent pentamer. The percentage of pentamer(+) CD8 T-cell remains at around 4% until the virus concentration decreases to 1.5×10^7 vp/injection.

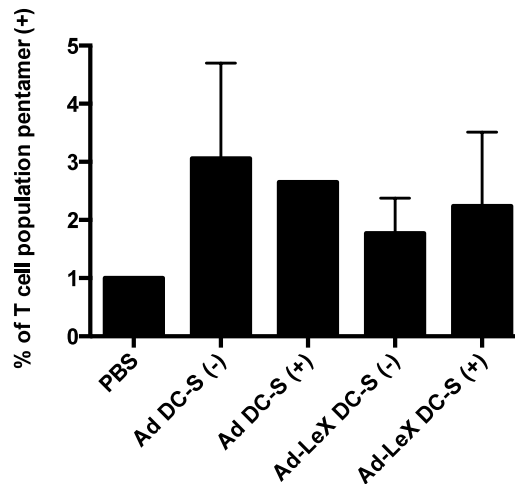


Figure 4 - tg hDC-SIGN(+) mice and DC-SIGN(-) littermates were injected with 1.5×10^7 vp of *ova*-Ad or *ova*-Ad-LeX. After 12 days, the mice were sacrificed and splenocytes harvested. The cells were then stained for CD8 and for anti OVA specificity using an OVA peptide-bearing fluorescent pentamer. There was no significant difference between any of the groups; around 3% of CD8 T-cells are OVA reactive. Bars represent the average of present biological repeats (n=3) and the errors bars are the standard error.

Female heterozygous hDC-SIGN transgenic (tg) mice were immunized with a mixture of both unmodified *ova*-Ad and luciferase-expressing Ad (*luc*-Ad) or *ova*-Ad-LeX and *Luc*-Ad-LeX, with identical boosts at 2 weeks. The mouse spleens were removed 3 weeks after the boost infections and the OVA-specific CD4(+) or CD8(+) T-cell activation assayed. Splenocyte cultures were pulsed with OVA protein or the immunodominant OVA-derived peptides for CD4(+) OVA₃₂₃₋₃₃₉ (MHC II) and CD8(+) OVA₂₅₇₋₂₆₄ (MHC I). T-cell antigen-induced proliferation, extracellular cytokine release and intracellular IFN- γ staining were then quantified.

Pleasingly, the most potent T-cell response resulted from the DC-SIGN(+) mice immunized with *ova*-Ad-LeX (**Figure 5**). Immunization of DC-SIGN(-)/littermate control mice with *ova*-Ad-LeX also significantly enhanced T-cell responses compared to *ova*-Ad immunized mice, potentially due to the reduction of viral neutralization and clearance. Another possibility is that alternative mouse lectins expressed on APCs could be selectively up-taking the LeX modified Ad⁷⁻⁹. Immunization using

ova-Ad-LeX resulted in an enhanced CD8(+) T-cell response relative to CD4(+) T-cell responses as shown by the frequency of IFN- γ producing T-cells upon re-stimulation with OVA₂₅₇₋₂₆₄ and OVA₃₂₃₋₃₃₉; this is consistent with a robust CD8(+) T-cell response to the viral delivery of the *ova* transgene. However, when the IFN- γ concentration is related to the number of OVA-specific T-cells, the amount of IFN- γ released per cell is higher in the CD4(+) T-cell population suggesting the elicitation of high-avidity CD4(+) T-cells. The high IFN- γ to IL-5 ratio indicates a robust Th1-type CD8(+) polarised immune response.

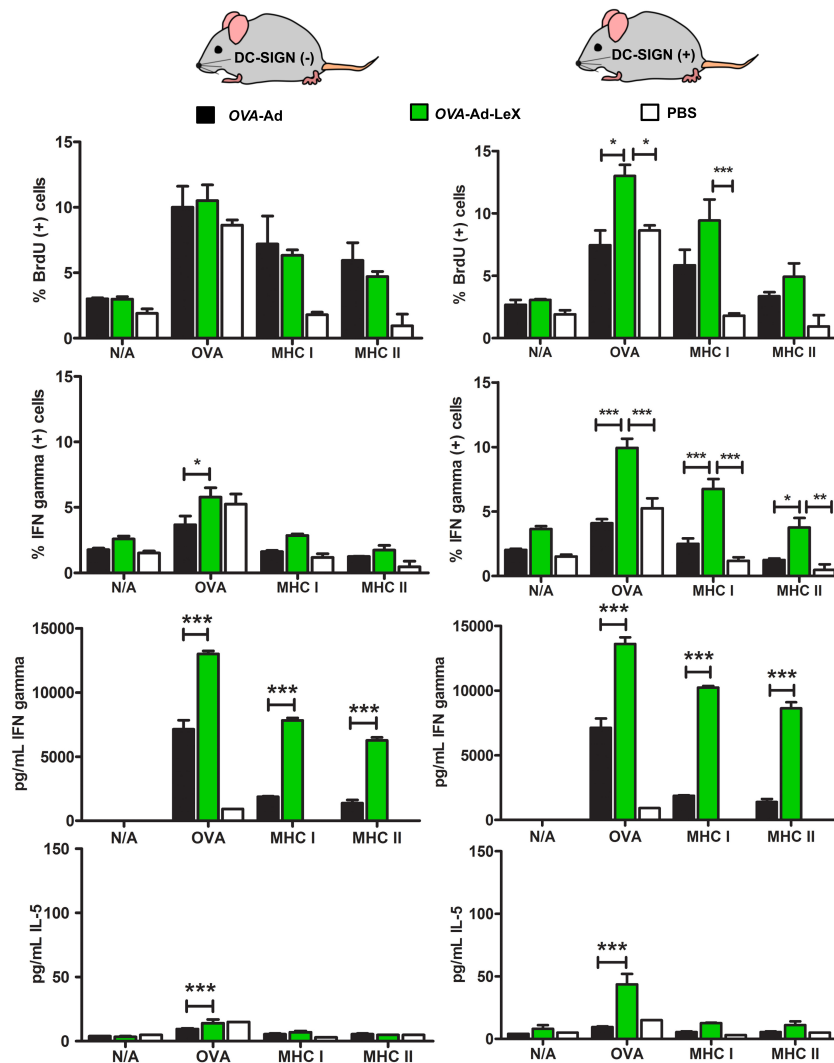


Figure 5. Splenocytes from tg hDC-SIGN mice immunized with *ova*-Ad-LeX released more IFN- γ and proliferated more upon stimulation with intact OVA protein and MHC I peptide. Splenocytes from Tg hDC-SIGN(+) or WT hDC-SIGN(-) mice, after

vaccination with either ova-Ad or ova-AdLeX, were cultured in the presence of OVA protein, OVA₂₅₇₋₂₆₄ (MHC I specific) or OVA₃₂₃₋₃₉₉ (MHC II specific) peptides. After 3 days, supernatants were measured for cytokine concentrations (c and d) and the cells analyzed for intracellular IFN- γ production and proliferation by BrdU incorporation (a and b). Statistics performed are 2-way ANOVA followed by Bonferroni's multiple comparison test where * $p < 0.05$; ** $p < 0.01$; **** $p < 0.001$.

6.1.2 DC-SIGN *in vivo* targeting

Investigations into the *in vivo* DC-SIGN targeting of the LeX modified vectors were conducted. Tg hDC-SIGN(+) mice and DC-SIGN(-) littermates (n=4), were injected I.M with luciferase expressing *luc*-Ad or *luc*-Ad-LeX; over a time course the mice were imaged for bioluminescence by intraperitoneal (I.P) injection with luciferin. The bioluminescence from all four groups decreased exponentially over 9 days. 14 hours after the boost injection (at 2 weeks) the bioluminescence measured was up to 80-fold that of after the initial Ad injection (**Figure 6**). Bioluminescence emitted from DC-SIGN(+) and (-) mice 24 hours after the first injection with *luc*-Ad was significantly greater than that from *luc*-Ad-LeX injected mice (10 fold). This could be a reflection of the switched and narrowed tropism as a result of the LeX modification. Ad possesses a naturally broad tropism and it is plausible that at the point of injection there would be many cell types available for infection. However, Ad-LeX is targeted towards hDC-SIGN and possibly mSIGNR-3 expressing cells, which may not be as abundant at the injection site. There were no significant differences between emissions from DC-SIGN(-) or (+) injected with *luc*-Ad-LeX at any time point.

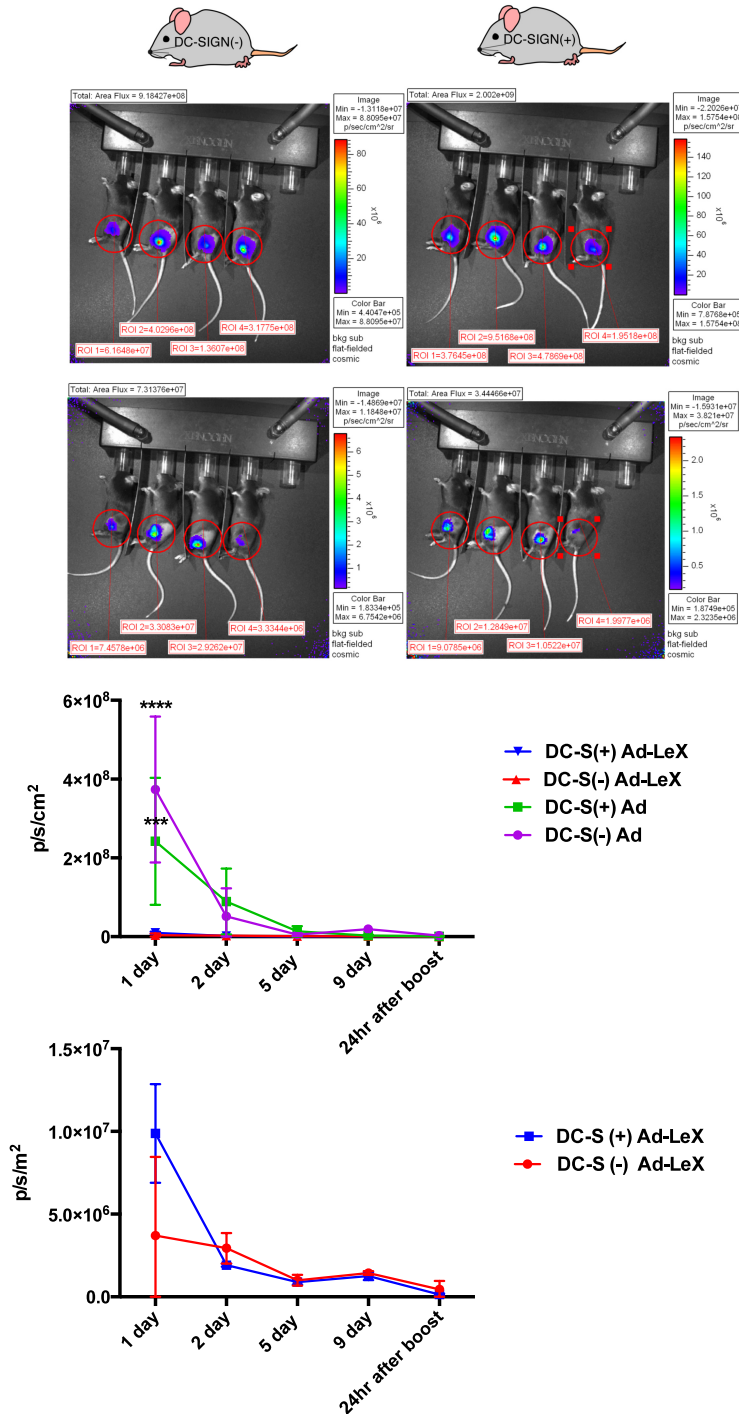


Figure 6 - Tg hDC-SIGN(+) mice and DC-SIGN(-) littermates were injected I.M. with *luc*-Ad or *luc*-Ad-LeX. The mice were then imaged for bioluminescence over a time course. *luc*-Ad in both mouse groups resulted in greater levels of bioluminescence at 24 hours. There is no significant differences in Ad-LeX transduction between DC-SIGN(+) and (-) groups at any time point. Data points represent the average of present biological repeats (n=4) and the error bars are the standard error. Statistics performed are 2-way ANOVA followed by Bonferroni's multiple comparison test where ** p < 0.01; **** p < 0.001.

In vivo DC-SIGN targeting was then investigated at the cellular level. A pilot experiment was run in 3 mice, with Ad modified with fluorescein isothiocyanate (FITC) in order to identify the draining lymph node of the I.M back leg injection site. After 4 hours the mice were sacrificed and the lymph nodes extracted and homogenised. It should be noted that there was some significant auto-fluorescence from the cells in the PBS-injected controlled mouse, most likely arising from macrophage populations^{10,11}. The FITC-labeled virus was mostly drained to the medial iliac lymph node and partially to the popliteal lymph node (**Figure 7a**). This is in agreement with the bio-distribution seen in the bioluminescence imaging of dissected mice (**Figure 7b and c**).

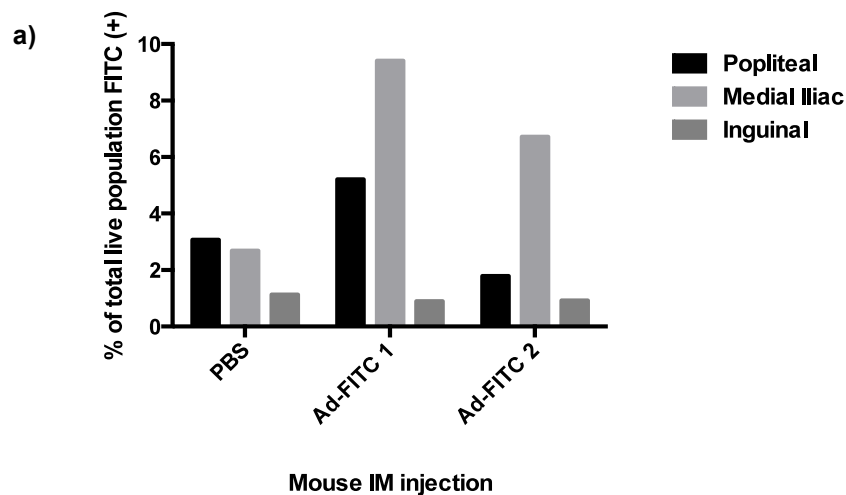


Figure 7 - a) Lymph nodes were extracted from two mice 4 hours after I.M. injection with Ad modified with FITC. The nodes were homogenised and the cells analysed for FITC by flow cytometry. Most FITC(+) cells were found in the medial iliac lymph nodes. **b)** tg hDC-SIGN(+) mice and DC-SIGN(-) littermates were injected with *luc*-Ad or *luc*-Ad-LeX. 24 hours later the mice were dosed I.P with luciferin, dissected and then imaged. The greatest bioluminescence was observed in the medial iliac nodes, followed by the proximal popliteal node. The Ad-LeX injections elicited less bioluminescence than Ad. **c)** Close up of a single mouse with the medial iliac lymph nodes extracted and placed to the left of the mouse. The proximal medial iliac lymph node is the primary draining lymph node of the I.M back injection site.



Following the *in vivo* tracking of the FITC labeled Ad, tg hDC-SIGN(+) mice and DC-SIGN(-) littermates were injected with *gfp*-Ad or *gfp*-Ad-LeX. After 24 hours the mice were sacrificed and the medial iliac lymph nodes extracted and homogenised. The cells were stained for DC (CD11c), macrophage (F4/8) and T-cell markers (CD3/19) and any GFP expression was analysed by flow cytometry (**Figure 8**). DCs and macrophage populations showed GFP expression in all of the groups; T-cell populations were not found to be GFP(+).

A significantly greater proportion of DCs and macrophages from DC-SIGN(-) mice injected with Ad were GFP(+) when compared to Ad-LeX injected mice of the same phenotype. This tropism switch towards DC-SIGN due to glycosylation could inhibit the Ad-LeX from infecting DC-SIGN(-) mouse cells. Unexpectedly, the number of GFP(+) cells in the tgDC-SIGN(+) mice injected with Ad was halved, compared to the DC-SIGN(-) littermates, for both macrophages and DCs. The reason for this is not clear, it is possible that the insertion of the transgene may have altered the ability of the mouse DCs and macrophages to internalize the Ad. However, the proportion of GFP(+) cells arising from Ad-LeX injection was equivalent to that of the Ad in the DC-SIGN(+) mice. This observation could be evidence of some DC-SIGN specific targeting if the resulting number of cells GFP(+) from the Ad injection, is taken as the non-targeted infection standard in DC-SIGN(+) or (-) mice separately. However, macrophages should not express the tgDC-SIGN. These results therefore are inconclusive at this stage and require further investigation.

An alternate explanation is that the cells measured were those that internalized Ad/Ad-LeX within the lymph, where the Ads may have been concentrated via non-targeted mechanisms. DC-SIGN specific targeting might therefore be more apparent in tissue resident DCs from the site of injection. Histological staining for DC markers and GFP expression could therefore reveal *in vivo* targeting.

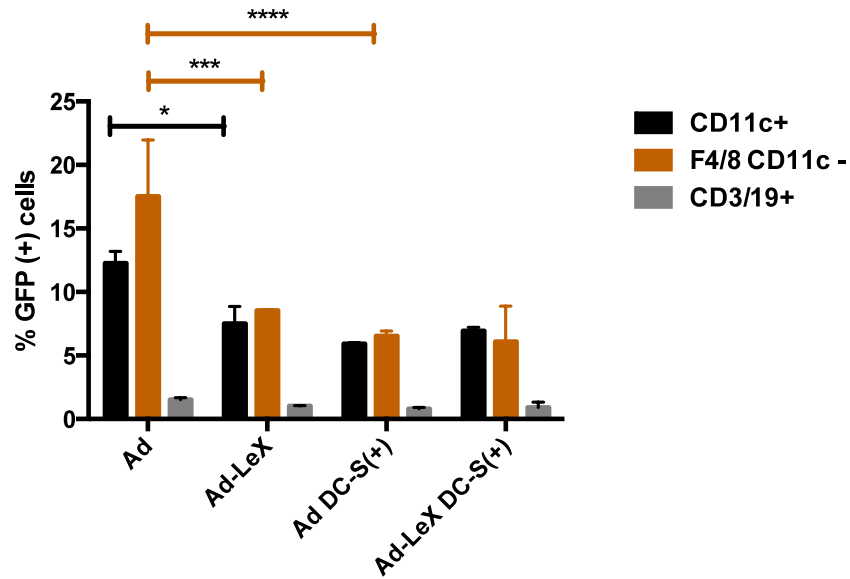


Figure 8 - The medial iliac lymph nodes of tg hDC-SIGN(+) and littermate DC-SIGN(-) mice were extracted 24 hours post injection with either *gfp-Ad* or *gfp-Ad-LeX*. The cells were extracted and stained with DC (CD11c), macrophage (F4/8) and T-cell markers (CD3/19) and analysed by flow cytometry for GFP expression. DC-SIGN(-) mice injected with Ad produced significantly more GFP(+) DCs and macrophages compared to Ad-LeX. The DC-SIGN(+) mice produced fewer GFP(+) cells from both Ad and Ad-LeX injections. Bars represent the average of biological repeats (n=3). Statistics performed are 2-way ANOVA followed by Bonferroni's multiple comparison test where * p < 0.05; ** p < 0.01; **** p < 0.001.

6.2 Discussion

T-cells from DC-SIGN(+) mice vaccinated with *ova-Ad-LeX* demonstrated enhanced rates of proliferation and INF- γ secretion, relative to T-cells from *ova-Ad* vaccinated mice. The high INF- γ to IL-5 ratio indicates a robust Th1-type CD8 polarised immune response. These results are consistent with the *in vitro* T-cell proliferation experiments, wherein a large increase in CD8 T-cell proliferation was recorded after stimulation by *ova-Ad-LeX* infected BMDCs. Such strong CD8 T-cell responses are considered to be necessary for successful vaccination against cancer and particular viral infections, each as HIV-1^{12,13}. Surprisingly, there was no increase in the numbers of circulating anti-OVA CD8 T-cells in the DC-SIGN(+) mice, despite this apparent enhanced response from *ova-Ad-LeX* vaccination. There was a

significant increase in T-cell INF- γ production from *ova*-Ad-LeX vaccinated DC-SIGN(-) mice, compared to *ova*-Ad. This could be due to the Ad-LeX being protected from mouse immune neutralisation and clearance mechanisms, as well as targeting to mouse LeX binding lectins. SIGNR3 and mouse MGL-1 show specificity for LeX and are expressed on mouse DCs, as well as other mouse cell types, such as macrophages⁷⁻⁹. These receptors could be enhancing DC uptake of the Ad-LeX, in the absence of human DC-SIGN.

There was no significant difference observed between anti-OVA antibodies titers from *ova*-Ad and *ova*-Ad-LeX vaccinated mice. This is again consistent with the *in vitro* results; limited CD4 T-cell proliferation was seen in response to *ova*-Ad and *ova*-Ad-LeX infected BMDCs.

In vivo tracking by bioluminescence revealed that the *luc*-Ad vectors resulted in more general infection, compared to *luc*-Ad-LeX. At the cellular level, GFP expression was higher in macrophages and DCs extracted from DC-SIGN(-) mice injected with *gfp*-Ad compared to *gfp*-Ad-LeX. Taken together, these results could demonstrate a narrowing of the tropism of the Ad-LeX.

Reflecting on the initial goals of this thesis, the chemical glycosylation of Ad with IME-LeX has produced a more effective DNA vaccine vector. The LeX modification narrows the tropism of Ad, abrogating the infection of CAR(+) cells and towards DC-SIGN(+) DCs. The sugar coating also shields the Ad from neutralizing antibodies and other host sequestration mechanisms. Together, these benefits appear to allow greater amounts of the injected Ad-LeX to infect DCs *in vivo*, generating an effective vaccination. Ad-LeX therefore could be translated into the clinic to generate strong CD8 T cell immunity. The narrowing of the tropism may allow lower doses of Ad to be used and may mitigate some of the toxicity issues associated with Ad therapy. Further studies could test the potential of the Ad-LeX as an anti-tumor vaccine. For example, Ad-LeX expressing a tumor antigen could be used to vaccinate mice that could then be challenged by a tumor graft. In a larger context, this work demonstrates the

feasibility of utilizing viral glyco-conjugates for the tailoring of immune responses, both for therapeutic and fundamental scientific applications.

6.3 Experimental

***In vivo* Vaccinations anti-Ad and OVA/Ad antibody ELISA.**

Heterogeneous hDC-SIGN and WT CD57BL/6 female mice (6-12 weeks) were injected I.M in the back left leg with a mixture of *Luc* and *OVA*-Ad or *Luc/OVA*-Ad-LeX viruses (3×10^9 vp/20 μ L, PBS). A boost injection of the Ads was administered at 2 weeks. At 4, 6 and 8 weeks tail bleeds were taken (20mL) and the sera isolated through centrifugation after the blood samples were left for 1 hour at room temperature to coagulate. Standard ELISA plates were coated over night at 4°C with unmodified Ad5 (1×10^9 vp/50mL /well), Ad-LeX Ad5 (1×10^9 vp/50mL /well), or OVA protein (20mL /mL) diluted in 50mM carbonate-bicarbonate buffer pH 8.5. Plates were then washed with washing buffer three times (PBS, 0.05% tween). The plates were blocked for 1 hour at room temperature (RT) with a 5% BSA, PBS solution (50mL /well) and then washed. Mouse sera were prepared in serial dilution in PBS, starting at 1:50, and 50uL were added to each well and left for 2 hours at RT. The plates were then washed and the IgG bound was then detected with anti-mouse IgG-horse radish peroxidase (HRP) (1 hour RT). After washing the extent of serum binding then visualized by the addition of 50uL TMB substrate. After 5-10 minutes, in the dark, a blue color developed and the reaction was stopped (50 μ L 1M HCl). Absorbance was then measured at 420nm.

Pentamer staining of T-cells. Tg hDC-SIGN mice (n=4) injected I.M in the back left leg with 20uL of *ova*-Ad/*luc*-Ad or *ova*-Ad-LeX/*luc*-Ad-LeX coated viruses with 1.5×10^9 , 1.5×10^8 or 1×10^7 vp of each virus *ova* and *luc* expressing Ad. 24hrs after vaccination the mice were injected I.P. with 200uL of luciferin (15mg/mL)(GoldBio), 8 minutes later the mice were imaged for bioluminescence (IVIS® bio-illuminometer). After a total of 12 days the mice were sacrificed and the spleens harvested. The organs were

processed into a single cell suspension mechanically, through a cell strainer and erythrocytes lysed (Life Technologies). The remaining lymphocytes stained with mouse anti CD3 (PE), CD4 (PerCp) (Biolegend), CD8 (FITC), and anti-OVA T-cell specific pentamer (APC)(Proimmune).

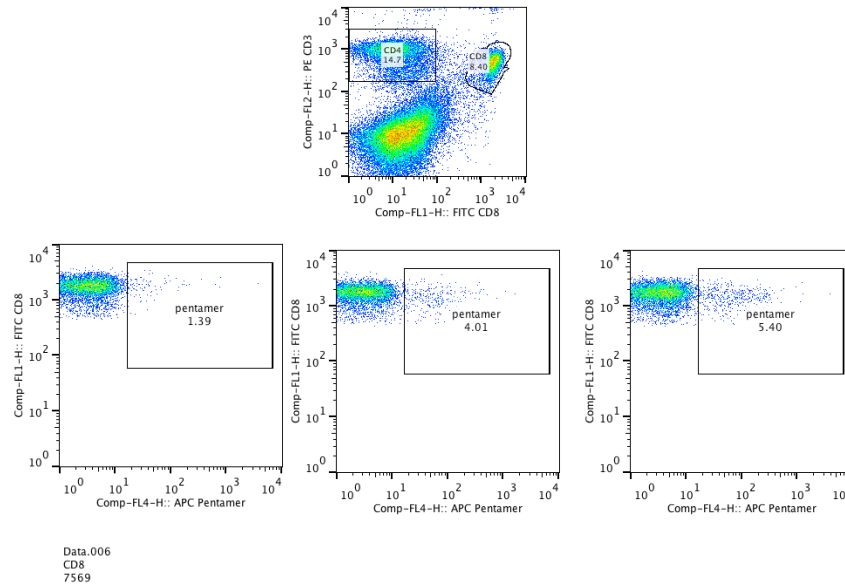
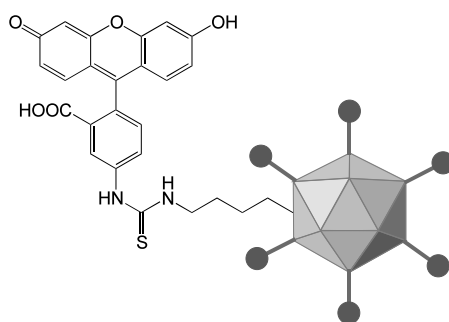


Figure E1 - Example of flow cytometry gating of splenocytes. CD3(+) CD8(+) T-cells were gated and the extent pentamer staining measured.

***In vivo* Vaccinations extra and intra cellular staining** Heterozygous hDC-SIGN(+) and littermate DC-SIGN(-) C57BL/6 female mice (6-12 weeks) were injected I.M in the left hind leg with a mixture of *luc*-Ad and *ova*-Ad or *luc*-Ad-LeX and *ova*-Ad-LeX viruses (3×10^9 vp/20 μ L, PBS 1:1 mixture). After 24 hours the mice were imaged to check for transgene expression. 200 μ L or luciferin (15mg/mL GoldBio) was injected I.P. and was left in incubate inside the mouse under anesthetic for 10 minutes. The mice were then imaged for bio-luminescence using an IVIS® bio-illuminometer. A boost injection of the Ads was administered at 2 weeks. 7 days post boost the mice were sacrificed and the spleens extracted. The organs were processed into a single cell suspension mechanically, through a cell strainer and erythrocytes lysed. The remaining cells were counted and plated (5×10^5 /well) in complete RPMI (RPMI, 50 μ M β mercaptoethanol and 10mM HEPES) and were left overnight. The splenocytes were then stimulated with

OVA protein (20 μ M, not endotoxin free, Invivogen), OVA₂₅₇₋₂₆₄ peptide or OVA₃₂₃₋₃₃₉ (20 μ M, Invivogen). After 24 hours supernatants were removed and extra-cellular cytokine concentration were measured by Luminex® bead assay (RnD sytems). Intra-cellular staining and BrdU incorporation was achieved through the addition of 0.1mM BrdU (BrdU kit eBioscience) before introduction of the antigens, then at 4 hours prior to harvesting Brefeldin A (10 μ g/mL, eBioscience) was added to wells. Intracellular BrdU and INF- γ staining was then conducted in accordance with manufactures protocol and the cells analyzed by flow cytometry.

Ad FITC



Luc-Ad (20 μ L, 2.08 $\times 10^{12}$ vp/mL) was dialyzed into 0.6M TAPS 4°C 2 hours. The solution pH was brought up to pH 9 just before the addition of the FITC with diluted NaOH. FITC (10 equivalents/lysine (22,000/vp)) was dissolved in 50 μ L of the TAPS buffer (pH 9) and added in portions to the virus solution and was allowed to react for 2 hours, RT with shaking. Next the virus was purified by dialysis into PBS HEPES 50 mM overnight at 4°C with two buffer exchanges. 10% glycerol was then added to the final volume and the virus concentration was measured by Picogreen assay (see previous). A control virus sample was run along side the Ad-FITC where the capsid remained intact. This sample was used as the background subtraction in the calculation of viral DNA concentration as the FITC may have altered the fluorescence. An infection on A549 was conducted to ensure the Ad-FITC was still infective.

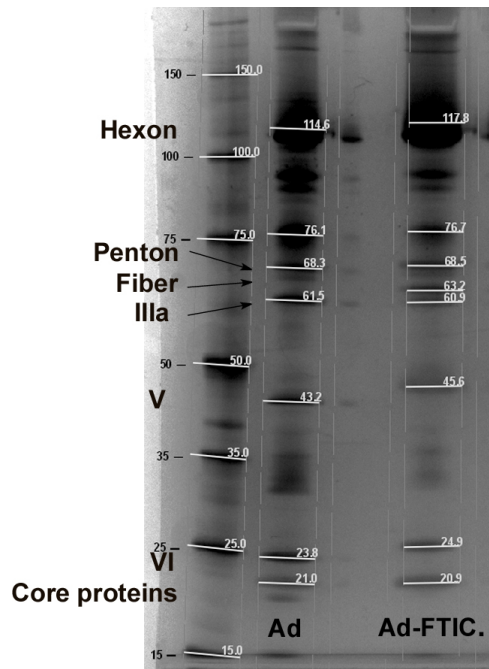


Figure E2- SDS-PAGE gel of Ad and Ad modified with FITC. Mass increases in the internal capsid proteins, hexon, penton and fiber and the internal protein V can be measured.

***In vivo* tracking of Ad-FITC.** A pilot experiment with Ad-FITC was conducted. Two female C57B/6 mice (6-8 weeks) were injected with Ad-FITC (3×10^9 vp/ 20 μ L PBS) in the back leg muscle. A control mouse was run with PBS only. After 4 hours the mice were sacrificed and the proximal popliteal, medial and inguinal lymph nodes were removed. The lymph nodes were then pushed through cell strainers and plated (1×10^6 /well) in a V bottomed 96 well plate. The cells were then treated with FC block for 10 minutes 4°C before staining 7-AAD live/dead stain (Biolegend): Panel 1- F4/80 PE, CD11c APC and 7-AAD live/dead stain. Panel 2 - CD3 and CD19 PE, Ly6G APC and 7-AAD live/dead stain. The cells were then analyzed by flow cytometry.

***In vivo* tracking of *gfp*-Ad-LeX and *luc*-Ad-LeX.** The *ova*-Ad-LeX/*luc*-Ad-LeX experiment was conducted with n=3 female tg hDC-SIGN; DC-SIGN(-) litter mates were also used. The mice received 3×10^9 vp of either *ova*-Ad-LeX/*luc*-Ad-LeX or *ova*-Ad/*luc*-Ad in 20 μ L PBS in the back left leg muscle.

After 24 hours the mice were imaged for luciferase expression (100 μ L Luciferin IP, 10min, then bioluminescence imaging using IVIS® bio-illuminometer, and sacrificed. The proximal popliteal, medial and inguinal lymph nodes were removed and then pushed through cell strainers and plated (1 $\times$ 10⁶/well) in a V bottomed 96 well plate. The cells were then treated with FC block for 10 minutes 4°C were removed and were stained with fluorescent antibodies (Biolegend): Panel 1- F4/80 PE, CD11c APC and 7-AAD live/dead stain. Panel 2 - CD3 and CD19 PE, Ly6G APC and 7-AAD live/dead stain. The cells were then analyzed by flow cytometry.

In vivo tracking of luc-Ad-LeX. Tg hDC-SIGN mice (n=4) injected I.M in the back left leg with 20 μ L of *luc-Ad* or *luc-Ad-LeX* coated viruses with 1.5 $\times$ 10⁹, 1.5 $\times$ 10⁸ or 1 $\times$ 10⁷ vp. 24hrs, 3, 5 and 9 days after vaccination the mice were injected I.P. with 200 μ L of luciferin (15mg/mL)(GoldBio), the mice were kept under anesthesia and their back legs were shaved. After 8 minutes the mice were imaged for bioluminescence (IVIS® bio-illuminometer). For dissection images the mice were sacrificed at 6 minutes post luciferin injection and quickly dissected and imaged within 4 minutes.

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