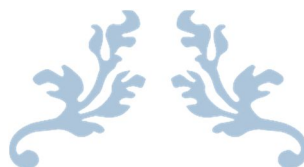


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Discovering novel immune-modulatory monosaccharides using high-throughput screening strategies



Meshal A. Alobaid

SUPERVISED BY

PROFESSOR AMIR M. GHAEMMAGHAMI

PROFESSOR MORGAN R. ALEXANDER

Preface & Acknowledgments

I start by praising God the almighty and Merciful for the graceful blessings bestowed upon me throughout my studies and my PhD journey.

Pursuing knowledge and conducting research is a passion I follow which have led me to this point where I submit my PhD thesis. This research project stemmed from my supervisors and my self's interests in finding cures for immunological diseases as the proverb states "Necessity is the mother of invention". This journey has guided me to understand the hardships and hurdles of being a researcher and the fruits of the labour you strive to gain. In truth I would have not been successful without the guidance of my supervisor Prof. Amir M. Ghaemmaghami to whom I am indebted and from his guidance I stand as I am proud of my achievements. I am also grateful to Prof. Morgan Alexander and our collaborators in Warwick University Prof. Matthew Gibson and Dr. Serah-Jane Richards for their guidance and input in my research. I would like to also thank my collages and friends who supported me and have been an addition to my every step forward. I am mostly indebted to my wife (eng.Bashayer Alqabandi), daughter and son (Leyan and Abdulrahman) who were able to stay by my side encouraging and supporting me through this journey, undoubtfully they have been my shining stars through this journey. I hold gratitude to my parents, my family, my friends and all whom helped me including Dr. David Onion and Miss. Nicola Croxall undeniable support in the flow cytometry department as well as Dennis Awuah for his help and support. I also acknowledge the opportunity given to me by my sponsors The Kuwait Civil Commission and the Ministry of Health to achieve this degree.

Abstract

Introduction: New strategies for immunomodulation and immunotherapy to fight infections, allergic diseases and cancer have shown real promise in recent years. Dendritic cells (DCs) are considered gatekeepers of the immune system and act as a bridge between innate and adaptive immune systems. Upon antigen uptake in peripheral tissues, DCs migrate to the lymph nodes and interact with T cells where they guide T cell polarisation towards distinct inflammatory or regulatory phenotypes. Their ability in shaping adaptive immune responses makes DCs ideal targets for immune modulation. The immune-instructive properties of DCs upon encountering T cells are dependent on their cytokine profile and the co-stimulatory molecules expressed. In this project, I used a high throughput screening strategy to investigate immune modulatory properties of a combinatorial library of immobilised synthetic monosaccharides with a particular focus on their ability to modulate key phenotypic and functional properties of human DCs. I also investigated the modulatory properties of these monosaccharides in particulate form, using gold nanoparticles (AuNPs) coated with monosaccharides, and studied their impact on DCs phenotype and function as well as identifying sugar moieties that enhance uptake of the AuNPs.

Results: A selection of immobilised carbohydrates including 100%1-amino-1-deoxy- β -D-galactose (Gal1), 100%2-amino-2-deoxy- β -D-galactose (Gal2), 90%2-amino-2-deoxy- β -D-galactose+10%1-amino-1-deoxy- β -D-mannose (Gal2-Man1), 2-amino-2-deoxy- β -D-galactose+10%2-amino-2-deoxy- β -D-mannose (Gal2-Man2), 40%1-amino-1-deoxy- β -D-mannose + 60%2-amino-2-deoxy- β -D-mannose (Man1-Man2) and 50%1-amino-1-deoxy- β -D-galactose +50%2-amino-2-deoxy- β -D-galactose (Gal1-Gal2), significantly downregulated LPS induced CD40 expression, compared to LPS alone, upregulated CD274 but had no significant changes in the expression of CD86 compared to unconditioned LPS stimulated

controls. This was accompanied by a significant decrease in the activity of key immune regulatory enzyme 2,3 indoleamine dioxygenase (IDO) and production of interleukin (IL)-12, a signature pro-inflammatory cytokine. Furthermore, DCs stimulated with the same combinations of carbohydrates showed a significant increase in production of prototypic regulatory cytokine IL-10. Collectively these data suggest an anti-inflammatory/regulatory phenotype for DCs treated with these carbohydrates. Furthermore, DCs conditioned with a selection of anti-inflammatory carbohydrates (Gal1-Gal2 and Gal1) induced naïve T cell polarisation towards regulatory phenotype while other carbohydrate combinations shown to increase IL-12 production (Man1-Man2, Gal2-Man1 and Gal2-Man2) induced T-helper 1 phenotype confirming the anti and prof inflammatory DC phenotypes respectively. Moreover, an increase in IL-17 and ROR γ t expression confirmed presence of TH17 in conditions treated with Gal1 and Gal1-Gal2 while a decrease in the latter was noted in Man1-Gal2. This was also confirmatory in the allergy module where Gal1-Gal2 maintained production of IL-17 and Man1-Gal2 had significant reduction in IL-17 clearly showing the carbohydrates potential in immunomodulation.

Carbohydrate coated AuNPs showed differential uptake by DCs where 100%fucose, 100%mannose, 90%mannose+10%Galactose and 80%mannose+20%Galactose coated AuNPs had highest internalization. Flow cytometry showed increased lysosomal and endosomal localisation for AuNPs coated with 100%fucose over the other conditions compared to uncoated AuNPs. Investigations of the fate of the particles showed significant increased co-localization of AuNPs with lysosomes for 100%fucose, 80%fucose+20%galactose and 70%galactose+20%mannose compared to other conditions. On the other hand, co-localization of AuNPs with endosomes for 100%mannose was significantly higher than other conditions. An understanding of receptor mediated endocytosis (active uptake) and micropinocytosis (passive uptake) mechanisms was assessed using Methyl- β -cyclodextrins where there was a significant reduction of uptake between

Methyl- β -cyclodextrins treated and untreated conditions suggesting the predominant mechanism of internalization of the AuNPs is active uptake.

Conclusions: Collectively these observations show the potential immunomodulatory effects of immobilised monosaccharides in priming DCs and skewing immune responses towards different functional pro- or anti-inflammatory/regulatory phenotypes. It also provides insights into using monosaccharides to optimize cellular uptake of nanoparticles as well as guiding trafficking towards different intracellular compartments. This understanding could pave the way for utilising simple monosaccharides in the development of anti-inflammatory coatings for graft implants and reduction of chronic inflammatory and autoimmune responses as well as potent drug delivery platforms guided towards antigen delivery and RNA silencing therapies.

Publications, Conferences and Awards

Publications from this work

Meshal A. Alobaid, Sarah-Jane Richards , Morgan R. Alexander, Matthew I. Gibson and Amir M. Ghaemmaghmi. Screening a combinatorial library of immobilized monosaccharides identifies simple sugars with immune modulatory properties (accepted for publication)

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Other Publications

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Dennis Awuah, Alisa Ruisinger, Meshal Alobaid, Chidimma Mbadugha, and Amir Ghaemmaghmi. MicroRNA-511-3p mediated modulation of the peroxisome proliferator-activated receptor gamma (PPAR γ) controls LPS-induced inflammatory responses in human monocyte derived DCs (Pending)

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25th June 2019, United Kingdom, Nottingham). Oral presentation at Life Sciences Research Symposium (11th-12th July 2018, United Kingdom, Nottingham)

Awards

- School of Life Sciences Travel Award
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Abbreviations

AhR Aryl hydrocarbon receptor

APCs Antigen presenting cells

AuNP Gold nanoparticles

BSA Bovine serum albumin

CTL C-Type lectins

DAMPs Damage-associated molecular patterns

DCs Dendritic cells

DC-SIGN Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

FBS Foetal bovine serum

FDA Food and Drug Administration

FOXP3 Forkhead box P3

GM-CSF Granulocyte macrophage colony-stimulating factor

HDM House dust mite

HLA Human leukocyte antigen

IDO Indoleamine 2,3-dioxygenase

IFN- γ Interferon gamma

IL Interleukin

LPS Lipopolysaccharide

MACS Magnetic cell separation system

MFI Median fluorescence intensity

MGL Macrophage galactose-type lectin

mDCs Myeloid dendritic cells

MHC Major histocompatibility complex

MR Mannose receptor

NPs Nanoparticles

PAMPs Pathogen associated molecular patterns

pDCs Plasmacytoid dendritic cells

PBA Phosphate-buffered albumin

PBMCs Peripheral blood mononuclear cells

PBS Phosphate buffer saline

PCR Polymerase chain reaction

PD-L Programmed death ligand

PRRs Pattern recognition receptors

ROR γ t RAR-related orphan receptor gamma

TCR T cell receptor

Th T helper

TNF- α Tumour necrosis factor alpha

TLRs Toll like receptors

T-reg T regulatory cell

Chapter 1:

General introduction

1.1 Introduction

1.1.1 Human immune system

The human body is continuously encountering foreign substances, microorganisms, and pathogens. This has resulted in the evolution of a sophisticated protective mechanism to be able to fight, repel and eradicate these invaders. The immune system is defined as a complex set of cellular and soluble (e.g. proteins and enzymes) components designed to protect the body against foreign substances, including infectious agents and tumour cells, while tolerating self-molecules [1]. The broad-spectrum of tasks that the immune system is involved in is essential to the host survival and is coordinated by immune cells which are distributed throughout the human body in areas such as blood, spleen, thymus, lymph nodes, skin, gut and bone marrow. The human immune system is divided into two major components, a none-specific immune component which is also referred to as the innate immunity and a specific immune component known as the adaptive immunity. Both arms of the immune system interact with each other to provide the full immune response that protects the human body against foreign substances while establishing self-tolerance against self-antigens [2].

The history of immunology started with Edward Jenner in 18th century when he discovered that cowpox “Vaccinia” can provide the human body with protection against the deadly disease smallpox. He developed the process to introduce an attenuated or weakened strain of the disease-causing agent to the human body to give them protection and called it vaccination. In the late 19th century Robert Koch was able to prove that there are causative agents for the diseases, and these are microorganisms. This led to a great advancement in the field of immunology combined with Edward Jenner’s vaccination method to develop vaccinations for different diseases to provide protection for humans against them. It was only later that the existence of antibodies was discovered by Bhiring and Kitasto through the work and research of the renowned immunologist Elie Metchnikoff [3].

Since then, great advancements have been made in the field of immunology both in our understanding of how the immune system works and also how this knowledge can be used to develop new strategies for the treatment and management of many diseases where dysregulation of the immune system is the main culprit [4-7].

1.1.1.1 Innate Immunity

The innate immunity is the first line of defence that encounters the infections and foreign substances. Upon interaction a non-specific immune response is initiated, which even on its own is capable of clearing many infections through killing pathogens [8]. The protection provided by the innate immune system can be divided into physical (for instance, provided by epithelial cells and skin) and chemical barriers, the action of leukocytes such as dendritic cells (DCs), neutrophils, macrophages and the Natural killer cells [9] and proteins in the plasma such as the complement system [10].

With regards to the leukocytes involved in the innate immune system, neutrophils and macrophages are involved in eliminating pathogens by the process of phagocytosis while dendritic cells are specialized in antigen uptake and presentation to the adaptive immune system [11]. Dendritic cells and macrophages are differentiated from monocytes or bone marrow derived progenitors and are capable of ingesting foreign substances into their cytoplasm neutralizing them [12]. These cells are typically resident in tissues and organs such as the respiratory tract, spleen, liver and lymph nodes where they acquire a tissue specific function as phagocytes. They can also be differentiated from infiltrating monocyte to a given tissue in response to infection, tissue damage or inflammation. The process in which phagocytes adhere and internalize foreign bodies is referred to as phagocytosis [13]. This process starts with migration of the phagocytes to the site of infection via attracting chemical substances called chemokines. Upon reaching the infected site the phagocytes opsonize to the infection causing agent which will then lead to the formation of pseudopodia from the outer

cellular membrane of the phagocytes [14]. The pseudopodia form around the infection agent creating a phagosome which is then engulfed into the phagocyte's inner cellular matrix. The phagosome then fuses with lysosomes to destroy its contents and neutralize them [15].

There are several proteins in the blood system that are considered part of the innate immunity. These proteins include but not limited to cytokines and the complement. Cytokines are soluble factors produced by cells in response to a stimuli to act upon same cell (autocrine effect) and other cells (paracrine effect) in order to activate, inhibit and/or attract other cells [16]. These substances are very potent and may elicit their action in small quantities. Cytokines can modulate immune cell functions, proliferation and induce a pro-inflammatory or anti-inflammatory effect. Examples of potent pro and anti-inflammatory cytokines include IL-12 and IL-10 respectively [17].

1.1.1.2 Adaptive immunity

The adaptive immunity also known as the acquired immunity is responsible for eliminating pathogens and neutralizing their toxins to protect the host [18]. Despite the close interaction and synergy between the innate and adaptive immunity, there are some key differences between them. Whereas innate immunity is non-specific, adaptive immunity has evolved specificity to the target pathogens. Also innate immune response does not have a memory and subsequent encounters with the same antigen are treated as the initial exposure with the same intensity and speed, while as part of an adaptive immune response memory cells are developed that accelerate the speed and intensity of immune responses against the same antigen upon subsequent exposures [19].

The adaptive immunity involves leukocytes referred to as lymphocytes and different types of lymphocytes are involved depending on the type of immune response. Lymphocytes descend from the lymphoid lineage from the bone marrow and migrate to the thymus to become T-lymphocytes or stay in the bone marrow and differentiate into B-cells through a very strict

selection mechanism [20]. The selection mechanism ensures that any lymphocytes that are able to recognise self-antigens are eliminated (central immune tolerance) or are efficiently suppressed (peripheral tolerance) without hampering body's ability to efficiently mount immune responses against foreign substances and pathogens. After differentiation, lymphocytes are able to circulate the body and migrate to lymph nodes where they reside. B lymphocytes are involved in the antibody-mediated immune response while the T lymphocytes are involved in the cell-mediated immune response [21].

1.1.2 Antigen presenting cells the link between the innate and adaptive immune systems

Antigen presenting cells (APCs) are considered as sentinels of the immune system and act as a bridge between innate and adaptive immune systems. Antigen presenting cells include cells such as macrophages, B cells and DCs [22]. Even though macrophages and B cells are able to present antigens to T cells they are not able to migrate to lymph nodes where most T cells reside. However, that is not the case for DCs, which are professional antigen presenting cells. DCs are abundant in any tissue where there is an interface with the external environment. These include all barrier tissues such as skin, lung epithelium and intestinal mucosa (Fig.1.1). DCs are also found in other organs such as spleen and peripheral blood, where they monitor the presence of foreign materials including pathogens.

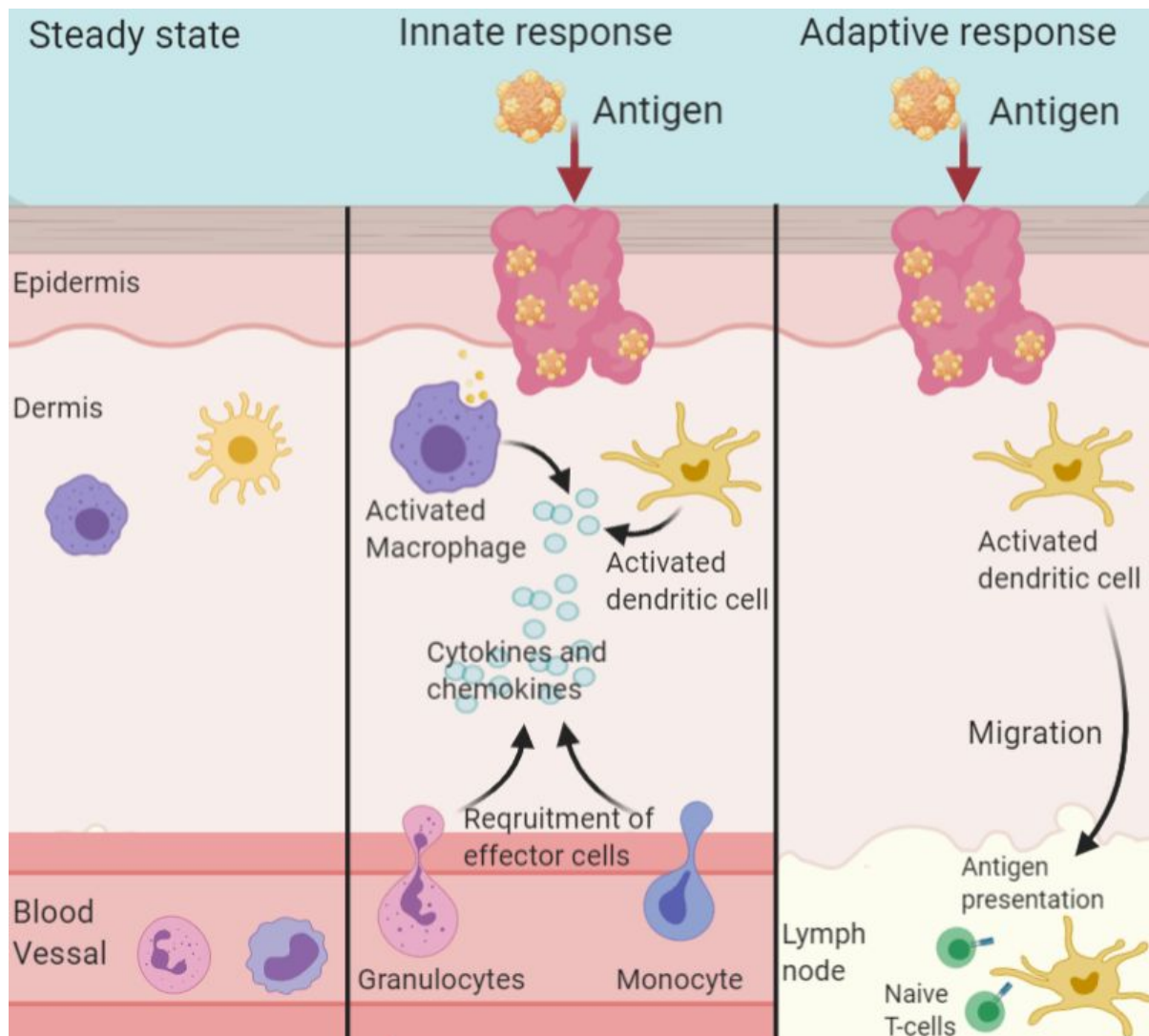


Figure 1.1: Innate and adaptive immune responses against foreign antigens. The innate immune system is the first line of defence after the physical barriers (skin). If the skin is penetrated, innate immune cells interact with the foreign antigen and engulf it in the cases of macrophages and dendritic cells in which cytokine and chemokines are secreted attracting more immune cells to destroy the foreign particles. The adaptive immune response starts when antigen presenting cells encounter the antigen and present it to T cells initiating a more specific immune response. This can happen in the blood vessels or in lymph nodes through migration of dendritic cells and antigen presentation and activation of the naïve T cells.

1.1.2.1 Dendritic cells

DCs originate from the bone marrow and can be either myeloid or plasmacytoid depending on the differentiation pathway (Fig.1.2). The myeloid DCs (mDCs) are also referred to as conventional DC as they are similar to monocytes and express the myeloid antigens CD11c, CD13, CD33 and CD11b [23]. Plasmacytoid DCs (pDCs) do not express these antigens, instead they are known by the expression of CD123, CD303 and CD304 antigens. DCs circulate the body as immature cells, characterised by low expression of co-stimulatory molecules and low level of MHC molecules on their surface. After interaction with pathogens they undergo maturation and subsequently migrate to the lymph nodes where they present antigen to T lymphocytes [24, 25] .

DC amongst other antigen presenting cells have been studied as a promising target for vaccination, drug delivery and immune modulation using synthetic particles with varying sizes, coatings as well as loading particles with drugs [26, 27]. Carbohydrates are known to be a ligand for CTLs and a mean in which cells interact with self or foreign cells which cause different responses depending on the type of carbohydrate, the ligand and the microenvironment.

1.1.2.2 Antigen recognition and uptake

Antigens that may be encountered by the immune system are vast and thus to execute the proper immune response each should be recognized accordingly. This occurs through pathogen recognition receptors (PRRs) where each is able to recognize specific types of antigens through their glycosylation patterns. Once ligation occurs between PRR and their carbohydrate counterpart, a series of reactions result in the outcome of the immune response. An example would be the Toll like receptors (TLRs) which are a family of receptors capable of recognizing pathogens through their special pathogen-associated molecular patterns (PAMPs) which are germline encoded molecular signatures that the innate immune system recognises as a threat.

The result would be an upregulation of the expression major histocompatibility complex (MHC) molecules bearing the antigen and co-stimulatory molecules in example CD80 and CD86 [28]. This enhances DCs function in antigen presentation and stimulation of the adaptive immune response.

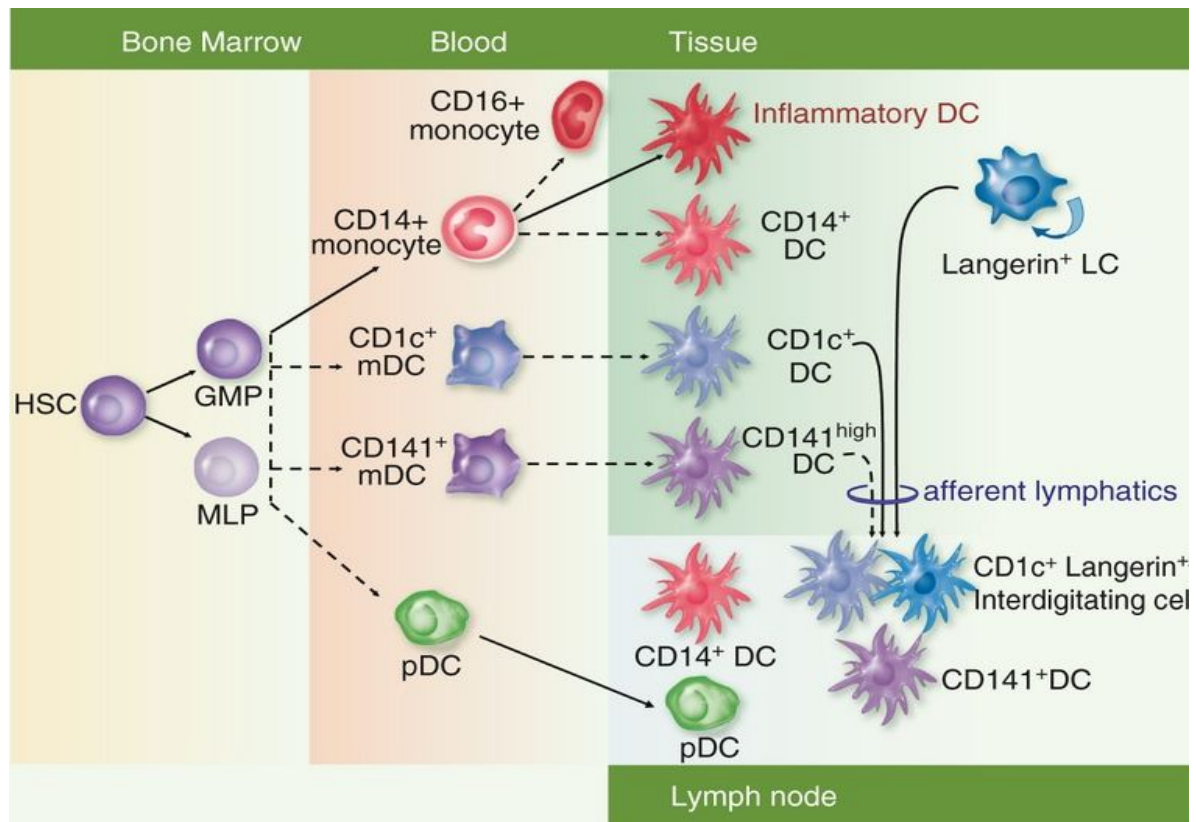


Figure 1.2: Hematopoietic lineages of dendritic cells with different subsets and surface molecules. DCs start from Hemopoietic stem cells (HSC) which differentiate towards Granulocytic-monocytic precursor or monocytic-lymphocytic precursor. Both cell subsets can differentiate towards the different types of known DC including plasmacytoid DCs (pDCs), monocytic DCs (mDCs) or CD14 monocytes that can further differentiate towards classical DCs. Adopted from [29].

1.1.2.3 The process of antigen presentation

The process of antigen presentation involves the activation of T cells in a series of steps initiating a cascade leading to the adaptive immune response (Fig.1.3). This process starts when DCs encounter foreign antigens through PRRs. The antigen is then recognised as either self-antigen, foreign antigen -pathogen associated molecular patterns (PAMPs)- or altered self -damage associated molecular patterns (DAMPs)-. PAMPs as the name implies come from pathogens that are invading the body, while DAMPs are generated after tissue injury and in some cases can be related to tumour cells. Examples of DAMPs are genetic materials (DNA and RNA) as nucleosides and hyaluronan fragments. In case of a non-self-antigen (possible infection) or altered self-antigen, the process goes on to capturing the antigen via PRRs and up taking it using phagocytosis. The ingested antigen is then processed via either the proteasome pathway or the endosome pathway [30]. In the case that the antigen is extracellular it will be processed via the endosomal pathway in which it will be phagocytosed forming a phagosome. The phagosome will then fuse with a lysosome which will digest the antigen resulting in a phagolysosome containing peptides. The phagolysosome will then fuse with a vesicle containing MHC class 2. The antigen will be attached to the MHC class 2 and the vesicle will move to the outer layer of the cell. The MHC and antigen will be presented on the cell surface.

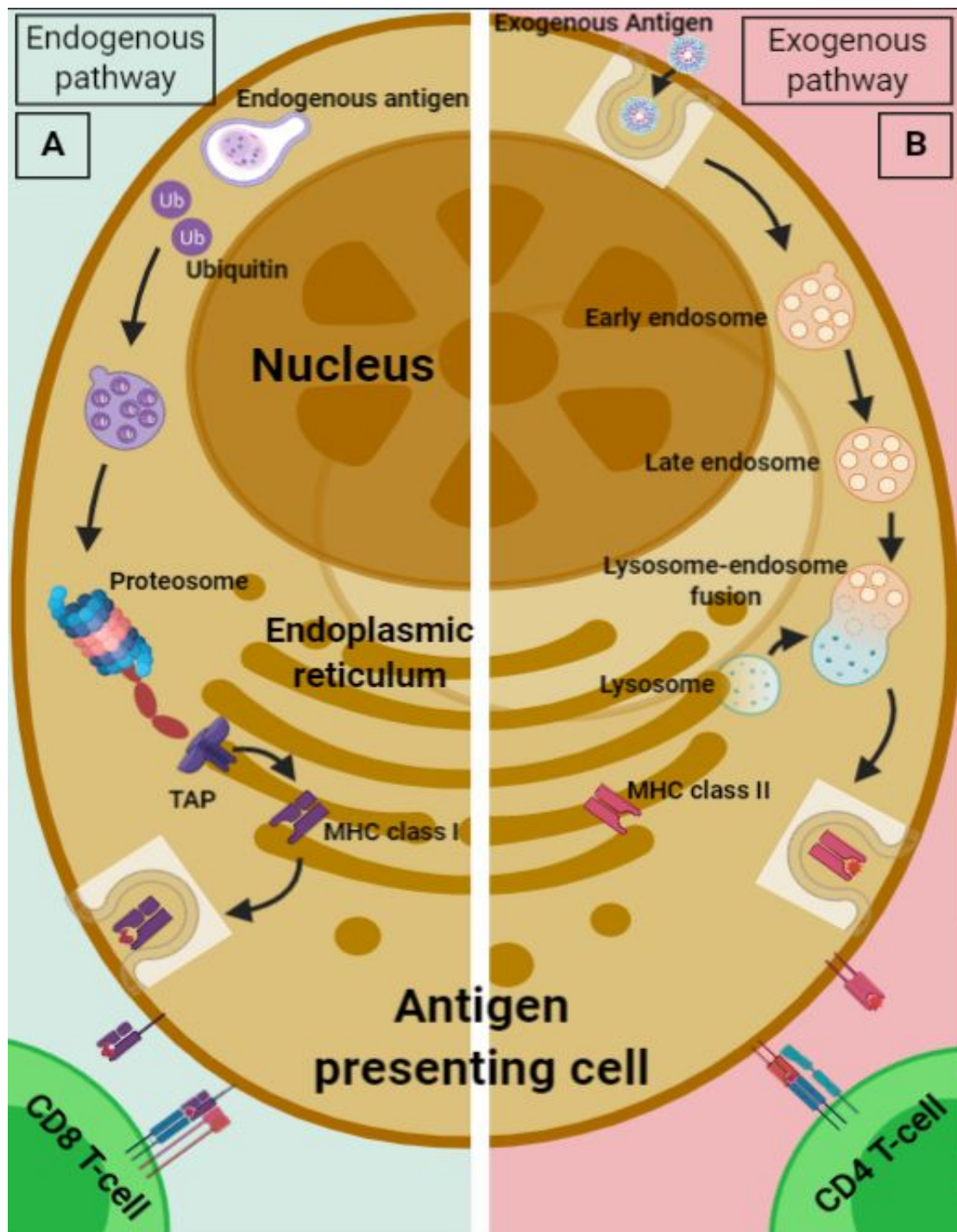


Figure 1.3: Dendritic cell antigen processing and presentation of exogenous and endogenous peptides. After recognition of foreign antigens and through phagocytosis and endosome is formed where it is fused with lysosomes and contents degraded. This fusion delivers degraded antigen to MHC molecules synthesised in the endoplasmic reticulum which travels to the cell membrane to present captured peptides.

In the case of intracellular antigens, the proteolytic pathway will be initiated where the antigens will fuse with a proteasome and get digested resulting in short peptides. These peptides will be transported to the rough endoplasmic reticulum to be attached to MHC class 1 molecules. The latter will then be packaged by the Golgi apparatus into a vesicle and transported to the cell surface where the MHC molecule will present the antigen. After the antigen has been processed and presented on the surface of the cell, the DC matures losing adhesion to tissue cells and migrating to lymph nodes [28]. DCs are then able to present the antigens to naïve cytotoxic (CD8) or helper (CD4) T cells to activate them depending on the MHC molecule expressed where MHC class 1 interacts with CD8 and MHC class 2 with CD4 T cells [31, 32].

In the case of self-antigens, the process goes through a none-inflammatory route unlike DAMPs and PAMPs. Self-antigens are presented by DCs to establish peripheral tolerance and suppress T cells that would show reactivity to self-antigens [33].

1.1.3 T cells subtypes and functions

After T cells are educated and undergo positive selection in the thymus, they are then able to circulate the blood and reside in lymph nodes where they await encountering an antigen through the process of antigen presentation. T cells mediating the adaptive immune response can be characterised into two main types depending on the expression of CD4 and CD8 molecules which are referred to as T-helper cells (Th) and T-cytotoxic cells respectively. CD8 Tc cells are able to act directly on the target cells killing them upon receiving antigens from APCs through MHC-I. This is done mainly for intracellular pathogens and tumour cells where target cells are identified by expressing aberrant or missing antigens and glycoproteins. On the other hand, CD4 Th cells have a more complicated job that requires it to further differentiate into different sub phenotypes each with different roles and functions. So far scientists were able to identify 6 sub phenotypes which are Th1, Th2, Th9, Treg, Th17 and T-follicular (Tfh) each carrying an important role in the immune response as described in Table.1.1 and can be

identified based on transcription factor expression and cytokine profile (Fig.1.4) [34].

Sub phenotype	Type of Stimulus	Function
Th1	Intracellular pathogens	Activates cell mediated immunity activates macrophages and complements to kill infected cells
Th2	Extracellular	Activates eosinophils, basophils, and mast cells to eliminate parasite; activates humoral immune system (B cells) to produce antibodies
Th9	Extracellular	Supports CD4 + T-cell expansion and survival; recruits mast cells (similar to Th2)
Th17	Extracellular pathogens	Recruits neutrophils and macrophages to the site of infection/inflammation
T-follicular helper (Tfh)	Extracellular/Intracellular pathogens	Resides mainly in spleen and tonsils, stimulates and sustains B-cell production of high-affinity antibodies
Regulatory T cell (Treg)	Extracellular/Intracellular Pathway activation	Regulates T-cell activation and proliferation through suppression. Prevents autoimmunity by suppressing autoreactive T-cells.

Table 1.1. The functions and defining activation characteristics of T cell subtypes. Each of the phenotypes is specialized to initiate or suppress the immune response depending on the type of pathogen or antigen.

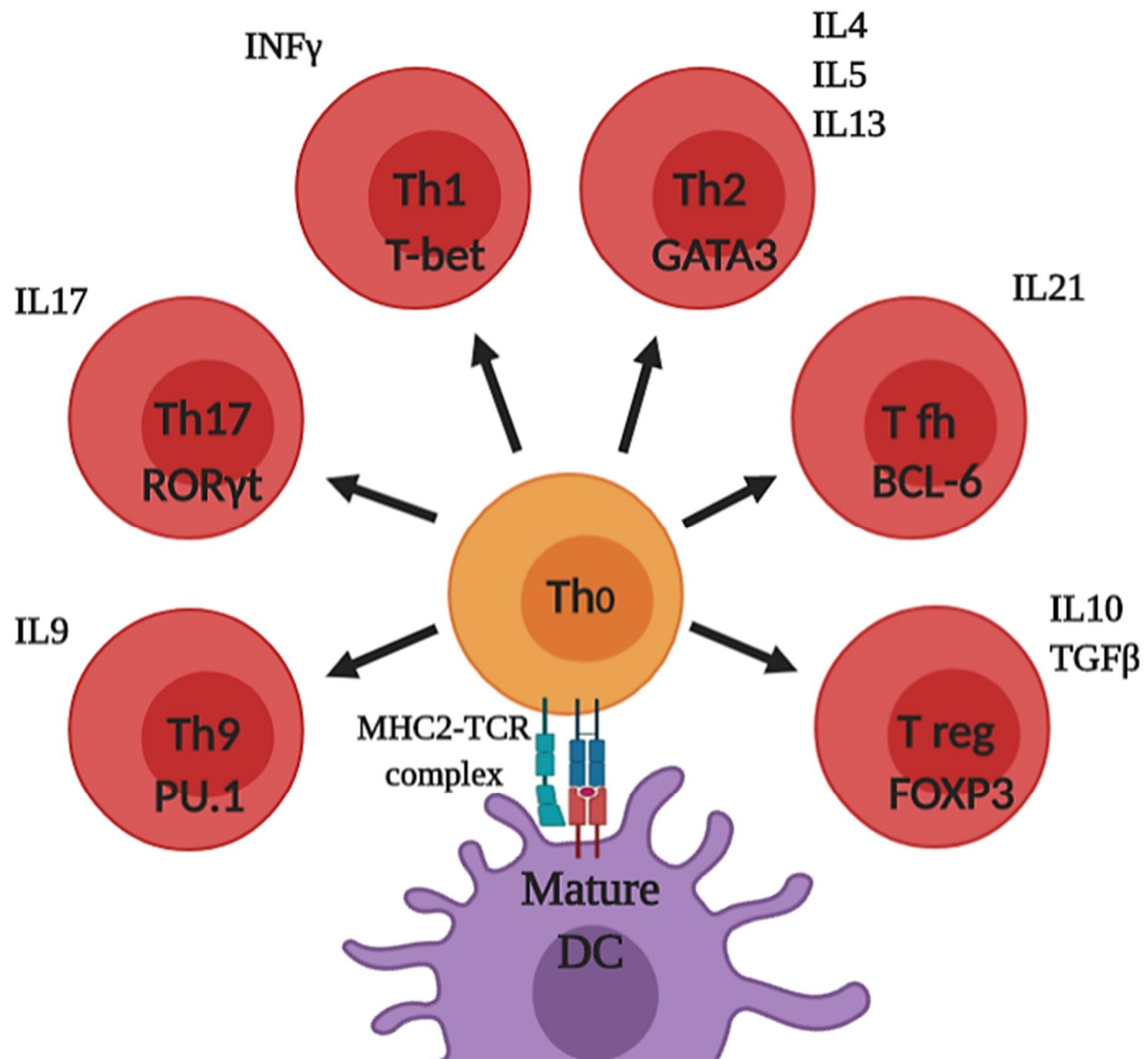


Figure 1.4: T-cell different subsets upon activation by mature dendritic cells bearing antigens. Each phenotype can be identified by expression of distinct genes and cytokine production in correspondence to its role in the immune response.

1.1.3.1 Immune tolerance

Immune tolerance can be defined as the host's ability to recognise self-antigens from non-self-antigens to not launch an immune response against own cells [35]. The immune system has many potent effector functions that when malfunctions in a disease state could cause serious damage to the host. Autoimmunity is one of the conditions that results from the disruption of the balance of the immune system causing the immune cells to attack self-molecules [36]. One of DCs' main roles is evaluating cells of the immune system to check for errors in their system that may pose a threat to the body and dispose of such cells while keeping viable ones to protect the body. This is done in a fascinating way where the DC are able to migrate to the thymus and bone marrow where T cells and B cells are maturing respectively. To tests these cells for immune tolerance, some DC present self-antigens on their surface while others present none self-antigens. Cells that react to self-antigens by binding strongly (high affinity) are ordered to induce apoptosis while others that bind in a low affinity (regulatory T cell) are allowed to survive in a process called negative selection. Another process involves cells that react to non-self-antigens where cells that bind in a moderate affinity survive while others that bind with low affinity would be ordered into apoptosis (positively selected) [37].

1.1.4 Action of Lectins

Lectins are a group of proteins that are specifically able to bind to certain carbohydrates and can be found in plants animals and microorganisms [38]. This group of proteins are used by cells to communicate with the outer environment or with other cells. For example, the outer membrane of the pathogenic *Enterotoxigenic Escherichia coli* contains lectins that recognise oligosaccharides on the surfaces of the gastrointestinal epithelial cells which in turn enables the pathogen to attach and secret a host of toxins causing stomach-ache and diarrhoea known as Traveller's diarrhoea [39]. Immune cells express lectins on their surfaces which mediates PRRs due to their ability to bind to the pathogen's polysaccharides and glycoproteins expressed

on their surfaces [40]. There are different types of lectins where some are transmembrane bound lectins while others are soluble depending on their roles [41].

Dendritic cells are immune sentinels that sense the surrounding environment using PRRs [42]. They are the only type of antigen presenting cells able to migrate to the lymph nodes and interact with naïve T cell population initiating an adaptive immune response [43]. DCs sample their environment consistently using PRRs which can be defined as conserved germ line coded receptors capable of recognizing foreign antigens from pathogens or aberrated self-antigens. This family of receptors include C-type lectins (CTLs), Toll like receptors (TLRs), Nod-like receptors, RIG-I-like receptors and Sialic acid-binding immunoglobulin-type receptors [44-47]. Each of the PRRs is able to recognize and bind different moieties (e.g. carbohydrates, DNA, RNA, etc) unique to pathogens initiating or dampening the immune response as illustrated in figure 1.5 [48]. In many cases different pathogens have evolved to exploit PRRs to their advantage. In this context there are many studies showing how pathogens exploiting CTLs to increase their survival and/or evade the immune system [49, 50].

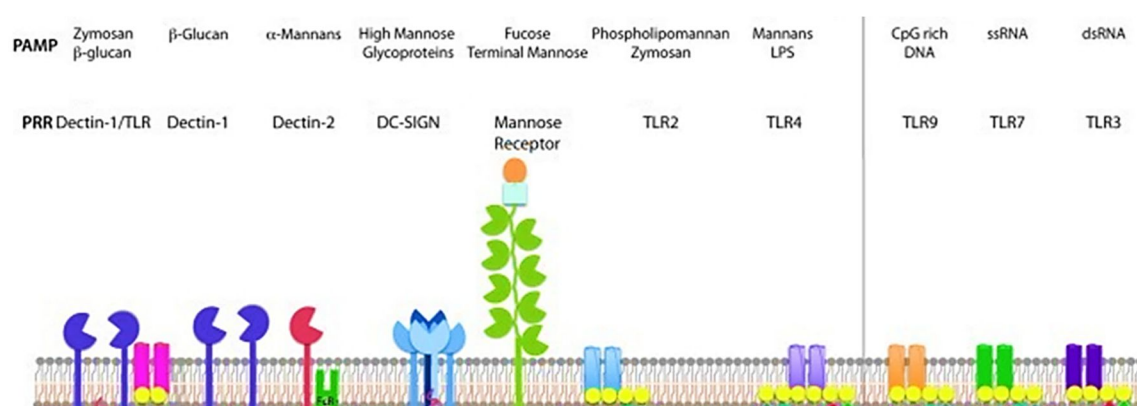


Figure 1.5: Different CTLs and TLRs and carbohydrate ligands. It can be noted that some CTLs share common glycan ligands which are mainly found on pathogens which aids recognition and cell activation. Adopted from [51].

CTLs can be membrane bound or soluble and on the cells surface of intracellular depending on their type and function [52, 53]. CTLs function in the presence of calcium and have affinities for different carbohydrates which can be found on pathogens or toxins and upon interaction would determine the response of DCs and ultimately guides the fate of the adaptive immune response (e.g. T cell polarisation) through production of different cytokines and expression of surface markers [54].

1.1.4.1 C-type lectins in pathogen and allergen recognition

C-type lectins generally are responsible for pathogen and allergen recognition depending on the type and source of antigens encountered. This family of PRRs require calcium to bind to carbohydrates (C-type) and are mostly found on cell surface [55]. CTLs work synergistically with other PRRs such as TLRs to modulate the immune response. An example of a lectin responsible for pathogen recognition is DC-SIGN (DC-specific ICAM3-grabbing non-integrin), a transmembrane C-type lectin. DC-SIGN is able to recognize mannose and fucose from extracellular or intracellular pathogens. Crosstalk between CTLs and other PRRs which get triggered simultaneously by the pathogen such as TLRs is vital to guide DCs response [56]. An example would be *Mycobacterium tuberculosis* or *mycobacterium laprae* where both pathogens contain mannose capped structures that are recognised by and bind to DC-SIGN initiating an intracellular signal [57].

The mannose receptor (MR) is another CTLs and is transmembrane receptor and like DC-SIGN, MR can be involved in active endocytosis through the clathrin mediated endocytosis. MR has been shown to be involved in modulating TLR signalling and outcome of the immune response. MR has been associated with allergen uptake and recognition of mannose moieties including self and non self-antigens. The interrelationship between MR, DC-SIGN and other PRRs could be quite complex where co-ligation of different receptors could lead to distinct immune responses. For example, our group have previously demonstrated a relation between

TLR4, aryl hydrocarbon receptor and indoleamine 2,3-dioxygenase axis where T cell polarisation skews towards Th2 where MR ligation modulates TLR4 signalling. This leads to IgE production against the allergen causing the signs and symptoms of the allergic reactions seen [58]. Macrophage galactose type lectin (MGL) also known as CD301 is a transmembrane type II receptor found mainly on macrophages and dendritic cells [59]. This receptor functions through recognizing monosaccharides galactose and N-acetylgalactosamine (GalNAc) as well as tumour Tn antigens [60]. MGL has been associated with pathogen evasion and tumour immunity through its function in tolerogenic DCs [61]. Also, it is capable of initiating TNF- α production when a TLR receptor is engaged as well as IL-10 production [62]. Studies have focused on this receptor to understand tumour immunity through engagement of the tumour antigens and the receptor on APCs . It has been also shown that MGL participates in the resolution of inflammations in neurons in conditions such Multiple sclerosis [63].

1.1.4.2 Cell to cell interactions

Lectins are important molecules in cell to cell interactions and play a role in many highly regulated processes such as cell migration, maturation and death. Upon antigen recognition by PRRs the process of maturation is initiated changing DCs phenotype to express a host of stimulatory and costimulatory molecules. The mature DCs are then able to migrate to lymph nodes where they present the captured antigen to T cells [64].

In regard to cell death or apoptosis, Selectins (S-type lectins) or galectins are known to play important roles. There are 15 galectins discovered as of now with only 10 of them have been identified in humans [65]. Galectins are synthesised by ribosomes in the cytosol and once synthesised can stay in the cytosol and regulate intracellular interactions. Galectins 1 and galectin 9 regulate apoptosis in the thymus where they are secreted by epithelial cell to induce apoptosis of T cells in the selection process. Another example is Galectin 3 where it has an

anti-apoptotic role in which it is responsible for inhibiting the release of mitochondrial cytochrome C which otherwise would cause apoptosis [66].

1.1.4.3 C-type lectins molecular mechanisms in recognition and binding of carbohydrates

The mechanisms in which CLRs are able to recognize and bind carbohydrates are dependent on the presence of Ca^{2+} ion [67]. The Ca^{2+} ion is important as it acts as a bridge between the CLR carbohydrate recognition domain (CRD) and the carbohydrate core monosaccharide [68]. CRD peptides offer six bonds to the Ca^{2+} ion while the carbohydrate offers 2 bonds through its OH group creating an octacoordinated binding with the Ca^{2+} ion and establishing a bond between the CRD and the carbohydrate. These bonds are relatively weak for an individual carbohydrate but CLRs are able to overcome this weakness through oligomerization and clustering of receptors. In this mechanism multiple CRD are in close proximity to each other in order to bind the epitopes on the carbohydrate creating a better affinity and avidity when binding to the monosaccharide cores [69].

DC-SIGN is a well-studied and established CLR and can serve as an example to illustrate the recognition and binding of a CLR to a monosaccharide core [70]. DC-SIGN consists of 4 major parts being the receptor's CRD, the neck region repeats, the transmembrane region and the cytoplasmic tail (Fig. 1.6). The CRD region is responsible for recognizing and binding monosaccharides in the presence of Ca^{2+} where a signal is generated [71]. To achieve better affinity and avidity like most CLRs, DC-SIGN oligomerize forming tetramers. This process occurs in the receptors neck domain where the helical repeats determine the oligomerization process [72]. This allows better recognition of pathogen monosaccharide repeats in which initiates a signal and in the process is internalized and presents pathogen antigens on an MHC II molecules. Aside from oligomerization, DC-SIGN also forms clusters on the plasma membrane

creating regions of high binding capacity and recognition of pathogens monosaccharide cores which serve to detect and internalize pathogens with increased avidity (Fig. 1.7) [73].

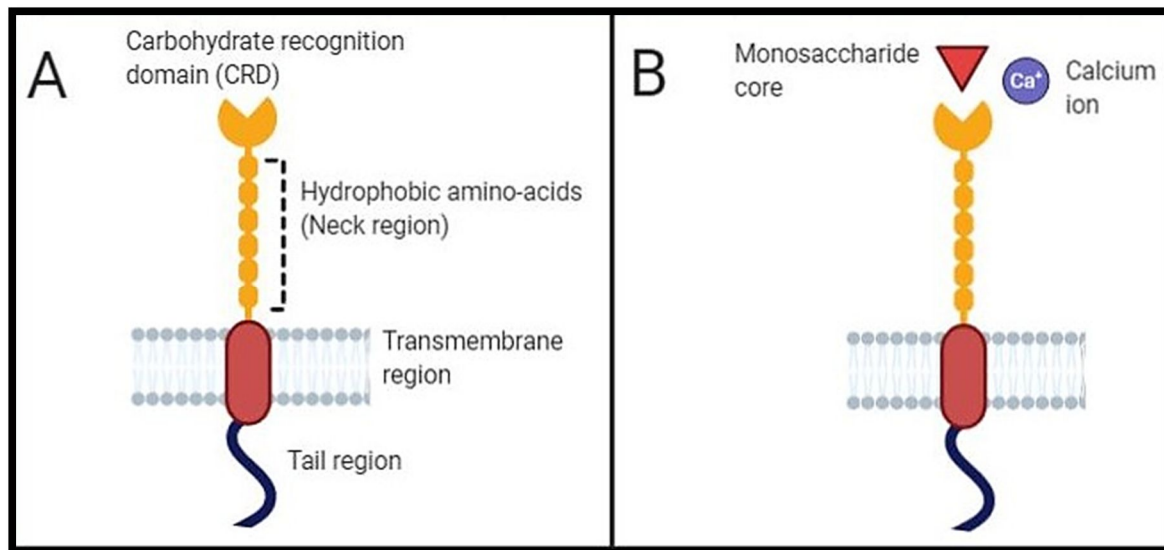


Figure 1.6: Illustrating the different regions of DC-SIGN as an example of CLRs and the binding of monosaccharide cores to the CRD in the presence of a Ca^{2+} ion.

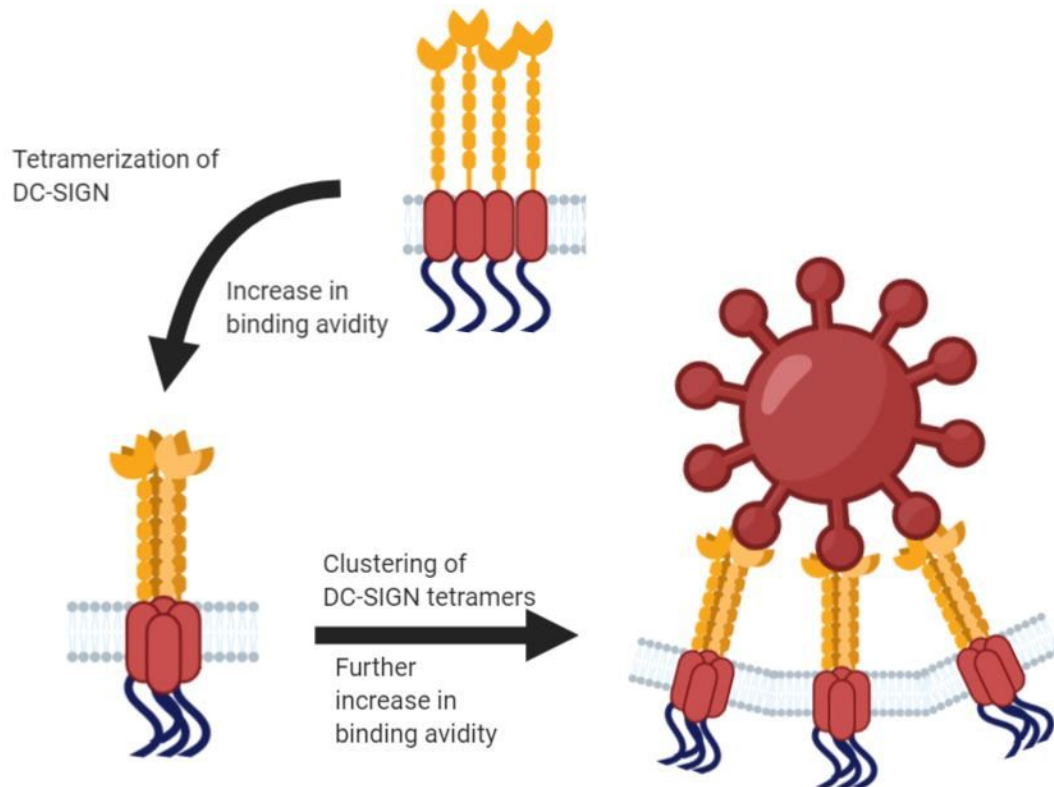


Figure 1.7. The oligomerization and clustering of DC-SIGN. The process of oligomerization of DC-SIGN into tetramers increases its binding avidity and the clustering of these tetramers on the cell membrane further increases cells ability to recognize, bind and internalize pathogens through their carbohydrate coating.

1.1.4.4 C-type lectin mediated Intracellular signalling in dendritic cells

Once a CLR is activated, an intracellular signal is initiated depending on the monosaccharide core of the antigen. DC-SIGN as an example is known to be bound to a protein called Leukocyte-specific protein 1 (LSP1) which is an F actin protein bound on the cytoplasmic surface of the plasma membrane. This protein alongside a complex of signalosome consisting of adaptor proteins KSR1 and CNK and kinase Raf-1 are recruited [74]. In the case of a mannose core PAMP such as mycobacterium ManLam or HIV, a Raft-1 dependent signal is initiated in which modulates TLR induced NF- κ B pathway through the acetylation and

phosphorylation of its P65 subunits [75]. This modulates the proinflammatory effect of NF- κ B fine-tuning the immune responses. In the case of a fucose core PAMP such as the LPS of *Helicobacter pylori*, the triad signalosomes are displaced from the cytoplasmic tail and only LSP-1 remains attached which in turn have modulates the TLR4 induced NF- κ B pathway and suppresses the immune response in the GI tract [76].

1.1.4.5 Immune modulation

DCs are able to modulate the immune response by interacting with T cells instructing their differentiation towards regulatory, pro-inflammatory or anti-inflammatory subtypes. As previously mentioned CTLs and TLRs are able to initiate a response when triggered individually or simultaneously and the response is different for each case. For example the interaction of fucose or mannose with DC-SIGN results in activation of the receptor which modulates TLR4 responses in DC as seen in *Mycobacterium* and *Helicobacter pylori* infections [76, 77]. Another example would be dectins which also belong to the family of the transmembrane CTLs and can identify fungal β -glucans initiating downstream effects leading to differentiation of T cells to Th17 the anti-fungal subtype alongside the pro-inflammatory Th1 [78].

DCs use different means for inferring their immunomodulatory potential using different means including cytokines, stimulatory and inhibitory surface markers and immune modulatory enzymes such as 2,3 indoleamine dioxygenase (IDO). IDO is a rate-limiting enzyme that converts L-tryptophan into kynurenine and in the process provides energy to the cell [79].

This can be seen when DCs encounter an infectious microorganism getting activated and matured, which has an increase in IDO activity leading to tryptophan depletion from its microenvironments. This hinders the growth of these infectious microorganisms as tryptophan is needed for the maintenance of their replication cycle. Moreover, studies show that IDO

contributes towards a pro-inflammatory response and is correlated with pathogenic inflammation related to tumour cells in which reduction of tryptophan and increase in kynurinine through highly active tumour cells leads to reduction of IDO activity in immune cells. This enables the tumour to avoid the immune systems effector cells facilitate tumour survival and metastasis [80]. Carbohydrates coating in microorganisms, parasites or even tumour cells seem to play a role in IDO modulation and thus their survival against immune system.

IDO modulation and inhibition has been implemented in cancer immunotherapies where IDO positive cancer cells are treated with IDO inhibitors and chemotherapy for a better outcome. These IDO drug inhibitors include epacadostat and indoximod which are in phase 2 clinical trials as well as navoximod which is in phase 3 of the clinical trials [9, 81, 82].

1.1.5 Carbohydrates and their role in the immune system

Carbohydrates are present on a diverse range of biological molecules through the process of glycosylation which in almost all cases lead to enhanced functionality of the biological molecule [83]. Carbohydrates are very abundant, varied, and essential for a living cell as virtually all proteins need to be glycosylated to be able to perform their functions with the exception of β 2-microglobulin. Also looking closely at both DNA and RNA they contain a carbohydrate group (ribose) that is essential for their structure and function [84]. Carbohydrates can be monosaccharides, single carbohydrates such as mannose, fucose, and arabinose or polysaccharides, multi carbohydrates ranging from 2-20 linked monosaccharides such as mannans, fucans, fucoidans and arabinans [85]. Some glycosylated proteins may comprise of up to 50% carbohydrates and loss of the carbohydrate group can result in fatal consequences for a living cell.

Carbohydrates have been known to be a means of cell to cell communication, adhesion and cellular signalling such as secretory glycosylated proteins (chemokines and cytokines) [86]. Also they provide structural stability to cellular proteins maintaining the way they fold which can be seen in the cell membrane [85]. Looking at the immune cells, they are decorated by cell receptors which are glycoproteins including PRRs such as CTLs and Toll-like receptors (TLRs), major histocompatibility complex classes I and II and many chemokine/cytokine receptors [87].

1.1.5.1 Types of carbohydrates and their linkage

Proteins and lipids are typically decorated by carbohydrates and such attachments are important to the function and recognition of the proteins or lipids components. These carbohydrate attachments are referred to as glycosylation and different types of glycosylation can be identified depending on the link to the conserved core structure [88]. Important types of glycosylation include N-glycosylation, O-glycosylation and glycosaminoglycans (GAGs) which are structured and ordered in a sequential manner due to the function of enzymes such as glycosyltransferase and glycosidase [89]. These carbohydrates attached components are able to bind to lectins and modulate cellular responses such as those found on microbial lipopolysaccharides (LPS) which are glycolipids of gram-negative bacteria including well known E-coli LPS and salmonella LPS used in research [90].

Looking at the glycosylation structures in Figure 1.8, N-glycans are linked to an asparagine protein residue with a subset of asparagine-X-serine/threonine while having a common conserved five sugar structure. O-glycans are linked to modifications of serine and threonine in a covalent bound. The common O-glycan is O-acetylgalactosamine which involve an α link between the β -D-N-acetylgalactosamine and the -OH of serine/threonine by an O-glycosidic bond. GAGs are unbranched and long repetitions of disaccharides structures and are an integral

part of Proteoglycans. They can also be present as free or unbound to a protein core such as hyaluronan. GAGs are highly sulphated increasing their capability to bind cytokines, chemokines and other factors in which is important for cell to cell interactions and homeostasis [91].

1.1.5.2 Carbohydrate clustering and oligomerization

As previously mentioned, living cells surfaces are covered with a wide variety of carbohydrates each in which contributes to the structural and functional attributes needed by the cells. These carbohydrates can form chains which can be linear or branched through either α or β glycosidic linkages to the hydroxyl groups of monosaccharides [92]. This enables carbohydrates to be in different forms such as free form e.g. found in hyaluronan or attached to either lipids or proteins such as glycolipids and proteoglycans/glycoproteins respectively (Fig. 1.8). This wide range of variation in carbohydrates forms contributes to intra and extra cellular interactions as well as pathogen recognition [93]. In order for these interactions to occur a state in which carbohydrates cluster forming unique structures “topologies” which then are recognised as specific ligands for the carbohydrates binding proteins. An example would be pathogen polysaccharides that form clusters and are recognised by the immune system [94].

Carbohydrate bind to their target protein with low affinity which in turn makes a bond of a single carbohydrate to a protein reversible. To achieve better binding and recognition of pathogens and cell to cell interactions some receptors have evolved to form clusters through oligomerization or display tandem repeats of multiple recognition domains [95]. This allows for multivalent binding of the clustered carbohydrates to their protein counterparts which are also oligomerized or present multiple domains of recognition creating a stronger binding capacity and thus proper cell interactions and recognition of pathogens [96]. MR is a good example where it can exist as monomeric or dimeric molecule of clustering of multiple CRDs [97].

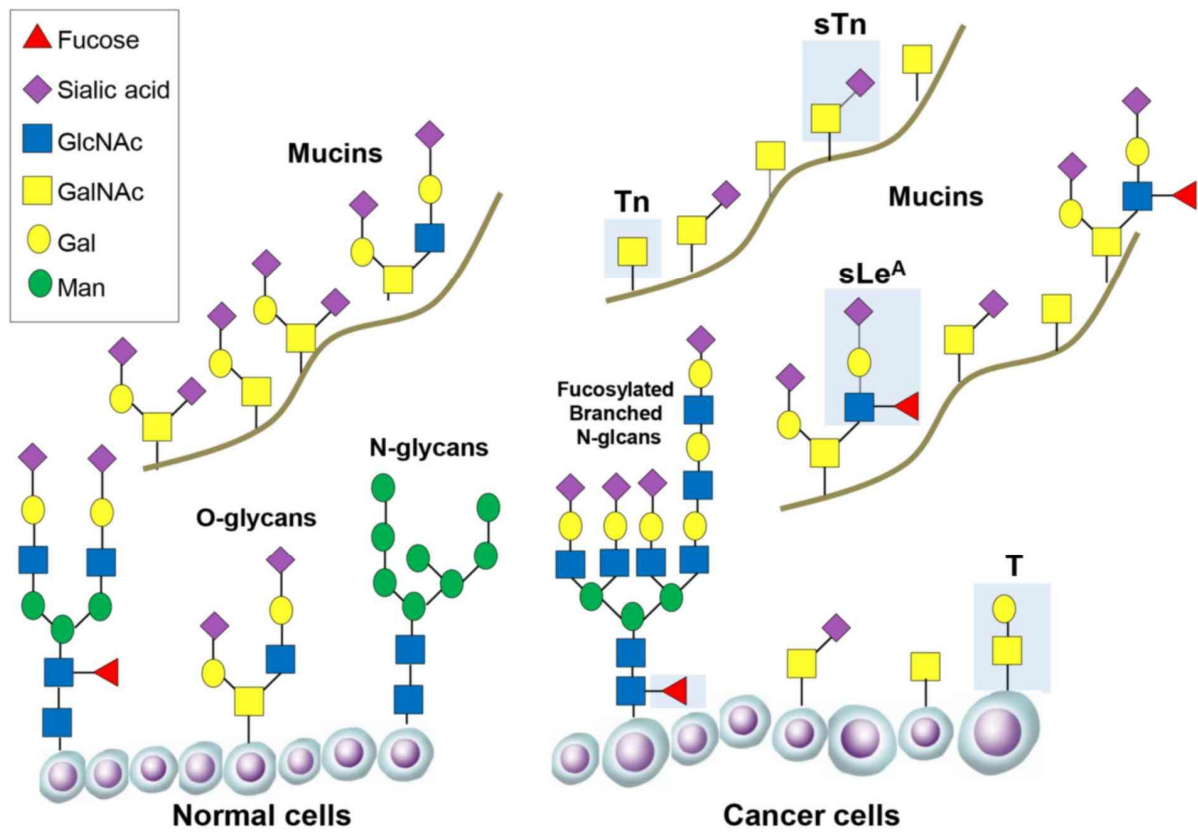


Figure 1.9: Different glycosylation patterns between normal and cancer cell. Over expression of aberrated glycosylation patterns such as Tn antigen and Lewis antigens can be recognized by immune cells and cancer cells killed in the process. Adopted from [102].

There have been many studies concentrating on the anti-tumour effects of carbohydrates and their benefits in developing vaccines against tumours. A study focused on the modification of a melanoma antigen 100gp with high mannose resulted in increased uptake of the antigen by DC-SIGN on dendritic cells and increase the antigen presentation to CD4 lymphocytes. This enhanced presentation shows possible methods where carbohydrates are candidates for use as anti-tumour vaccines [103].

Furthermore, some studies have offered promising results in targeting certain biomarkers using carbohydrates ligands instead of antibodies. Since some tumours express known altered markers, carbohydrate ligands can be synthesized to bind to them and deliver drugs to the tumour cells. For example, sialic acid binding immunoglobulin like lectin (Siglecs) has been studied as a promising route for drug delivery to cancerous leukocytes and lymphocytes involving CD22, CD33, Siglec-7 and Siglec-9 of the Siglac family [104].

Studies in the glycoimmunology field have been focusing on the immunomodulatory aspect of polysaccharides while much less is known about potential immunomodulatory potential of monosaccharides.

1.1.6 High throughput screening strategies to study immune modulatory properties of monosaccharides

High throughput screening strategies used in biology and drug discovery enables the screening of a large number of compound libraries to a biological or biochemical target [105]. These methods facilitate the identification and validation of compounds of interest that target various factors for example in diseases [106]. Studies have shown promising outcomes in the use of glycan as immobilized coatings or as attached to polymers to enhance targeted uptake or modulate cells [107].

Immobilized monosaccharides are able to interact with target cells without being engulfed giving us the understating of the immunomodulatory properties of the monosaccharide on a

surface bigger than the cells endocytic capacity while particulate carbohydrate coated gold nanoparticles will give us a better understanding of the monosaccharide ability to attract endocytosis and antigen presentation [108]. Each of the methods is taken into prospect to address different kinds of treatments and management of diseases such as immobilized monosaccharide would aid in development of anti-inflammatory coatings for implants and surgical equipment while particulate monosaccharides include therapies towards drug delivery for cancer immunotherapies and vaccinations.

1.1.6.1 Immobilized carbohydrates

Immobilization of carbohydrates to different surfaces has been studied to understand both the surface chemistry and the physical properties of the carbohydrates in modulating immune cells towards pro-inflammatory or anti-inflammatory or mixed responses [109]. Presently the use of immobilized carbohydrates has been focused on because of its use in long term or permanent implants and short-term biodegradable implants as well as engineering tissue scaffolds and coating invasive intravascular devices with immunomodulatory surfaces to provide anti-inflammatory responses lowering complications to the hosts. For instance, it has been shown by Hotaling *et al.* that adsorbed carbohydrates with bovine serum albumin influence DC responses in relation to the density and composition of the carbohydrate.[110] However, there are limited studies looking at immunomodulatory properties of monosaccharides with different combinations.

1.1.6.2 Nanoparticles

Nano scale particles have attracted attention due to their small size range (1-100nm) compared to microparticles giving them advantages in delivery routes (systemic, oral or inhaled) and physical stability as they do not aggregate like microparticles. Polymers used in drug delivery have to be biocompatible and are small enough to be excreted by the host system as to not

accumulate in organs causing toxicity to the host. Nanoparticles (NPs) can be synthesised from a wide range of materials which attributes to the immunomodulatory properties of the NPs. There are several biological and non-biological materials used to synthesise NPs in example for biocompatible such as polyglycolic acid (PGA), which is often used in tissue engineering scaffolding, metals such as gold, which are used for drug delivery and others such as glass and hydrogels used in manufacture implants and medical devices. NPs are widely used for targeted drug delivery and imaging in different organs and tumours [111, 112]. One of the issues is targeting specific organs or intracellular [113].

Different strategies have been used to enhance these properties and using coating with carbohydrates to direct delivery to particular tissues or cellular compartments is one of such strategies [114].

1.1.6.3 Gold nanoparticles

Gold is an inert and stable metal thus not having the potential to react and cause harm to the host. Many studies have looked at gold nanoparticles (AuNPs) as means of drug delivery and imaging techniques where target cells are promoted to uptake the particles through modification of particle surfaces in example antibodies or saccharides. AuNPs of sizes >20nm can be cleared by the kidney which makes that range of size less likely to cause accumulation toxic effects and more favoured towards immune-therapies [115, 116]. A study looked at oral gold nanoparticle suspension toxicity concluded that nanoparticles of 50nm in diameter are not toxic until a dose greater than 5000mg per kg of weight is reached where at this point most substances are likely to have a toxic effect [117]. Another study showed promising outcomes in tumour targeted treatment using PEGylated AuNPs [118]. Recent studies explored polysaccharide carbohydrate coatings of AuNPs and data showed how cells uptake behaviour change according to the type of carbohydrates used and the concentration of the functionalized carbohydrates [119].

1.1.6.4 Drug delivery mechanisms involving gold nanoparticles

There are several factors to consider when choosing nanoparticles for drug delivery aside from biocompatibility a) drugs need to be released into the site of treatment b) the body distribution and affinity of the particles to the target cells and c) the clearance of the nanoparticles by the body of the drugs released (shelf-life of nanoparticles and half-life of the drugs) [120]. Biodegradable polymers have been favoured for their low toxic effect. However, considering that non-biodegradable polymers are more stable both physically and chemically and have a longer shelf life, which is needed for most treatments including tumour immunotherapies, it will provide a more suitable delivery means. This is attained by conjugation of the AuNP with peptides, antibodies and/or carbohydrate moieties depending on the therapy and target cells intended [121]. For example targeting a tumour cell directly would require the use of antibody conjugated nanoparticles while vaccinating against tumour would require use of carbohydrate moieties to enhance uptake by the target immune cells and the tumour peptides to promote MHC expression and activation of T-cells against the tumour-bearing peptides [122].

1.1.6.5 Gold Nanoparticles in tumour immunotherapies and vaccinations

Tumour immunotherapies have been promoted in the treatment of tumours as safer alternatives or in combination to chemotherapies. The use of AuNPs as means for delivery of tumour antigens, adjuvants and modulation of dendritic cells or T cells enabled new methods not only for treating cancers but also for vaccinations against them [123]. Mounting an efficient and robust immune response will provide clearing of the tumour cells and protection against it with the host's memory cells. AuNP particles as drug carrier systems are convenient to present tumour and pathogen antigens to immune cells without having an effect themselves on cell phenotype. For instance, such approach was used recently in development of a novel vaccination method where neoantigens and nanoparticles in ~20nm size successfully delivered

antigens to APCs and subsequently activated CD8 lymphocytes [124]. This shows that the addition of drugs or pro-inflammatory adjuvants to stimulate the immune cells and promote cellular uptake and activation against the presented antigen therefore host immunity can be achieved. Also targeted therapies towards tumour cells by coating AuNPs with antibodies specific for tumour cell glycoproteins and drugs that aid in detection or destructions of these cells by attracting and reprogramming immune cells against or activating clearance mechanisms in the spleen and lymph nodes to eradicate tumours. Therapeutic efficiency of coated AuNPs has been shown in a number of studies however most are still towards clinical trials and not yet used as mainstream methods to combat cancers or pathogens through routine vaccinations. Other therapies including modulation of the cancer microenvironment and gene therapies using AuNPs as delivery system have also been attempted and are still under research. However, most recent therapies include personalised vaccine and tumour therapies where each individual is presented with customised AuNPs depending on the tumour antigens and glycoproteins and stage and type of tumour [125].

1.1.7 Uptake Mechanisms of coated and uncoated gold nanoparticles

Cellular uptake and internalization of different materials through engulfment by being surrounded with cell membrane components is referred to as endocytosis. There are different types of endocytosis including pinocytosis, phagocytosis and receptor mediated phagocytosis, each of which leads to vesicle formation and internalization of the materials. Pinocytosis is a means of uptake in which cells increase in volume through internalizing fluid to maintain balance as well as internalization of important components for cellular metabolism. Small inactive particles maybe be ingested through this process however this is not of main interest as it is unspecific and untargeted. Receptor mediated phagocytosis is the main method of uptake for drug delivery as they are more targeted and towards interaction and cellular activation. Uncoated AuNP of sizes less than 20nm are shown to be internalized by immune cells and

penetrate solid spheroid tumours much more efficiently than $>50\text{nm}$ attributing to the fact that they are internalized non-specifically through micropinocytosis [126-128].

Coating AuNPs with antibodies or glycoproteins has shown targeted delivery of these particles to immune cells through receptor mediated endocytosis. This facilitates drug and antigen delivery to immune cells for vaccination or cell modulation [129].

1.1.7.1 Micropinocytosis

Micropinocytosis is a type of endocytosis involving actin mediated membrane to form pods on the cell membrane surrounding and retracting substances into the cell. This is commonly used by cells to engulf nutrients and maintain osmotic pressure and is initiated by growth factors such as that of macrophage colony stimulating factor 1 and epidermal growth factor [130]. However, for antigen presenting cells it also serves as means of continuously surveying the environment for any non-self-antigens. The process starts with cell membrane pods being formed “lamellipodia” which folds back into the cell creating an irregular vesicle “micropinosome” (Fig.1.10). The micropinosome can be distinguished from other vesicles that they are not coated unlike endocytic vesicles creating through the clathrin pathway and are larger up to $5\mu\text{m}$ in size. These endosomes are then matured from early to late endosomes and fused with digestive enzyme-containing vesicles “lysosomes” for degradation. Antigen presenting cells are able to express peptides of the antigens on the MHC to lymphocytes initiating the adaptive immune response [131].

1.1.7.2 Clathrin-mediated endocytosis

The clathrin-mediated endocytosis (CME) is a receptor orchestrated uptake of particles of $\sim 100\text{nm}$ in diameter. These particles are enclosed in a protein coated pit which is initiated by a set of proteins including the clathrins ultimately causing the assembly of lattice like structures and the formation of the endocytic vesicles inside the plasma membrane of the cell (Fig.1.11).

These particles carrying vesicles are then trafficked into the cells where they fuse with lysosomes for degradation and go through the routes of antigen processing and presentation [132].

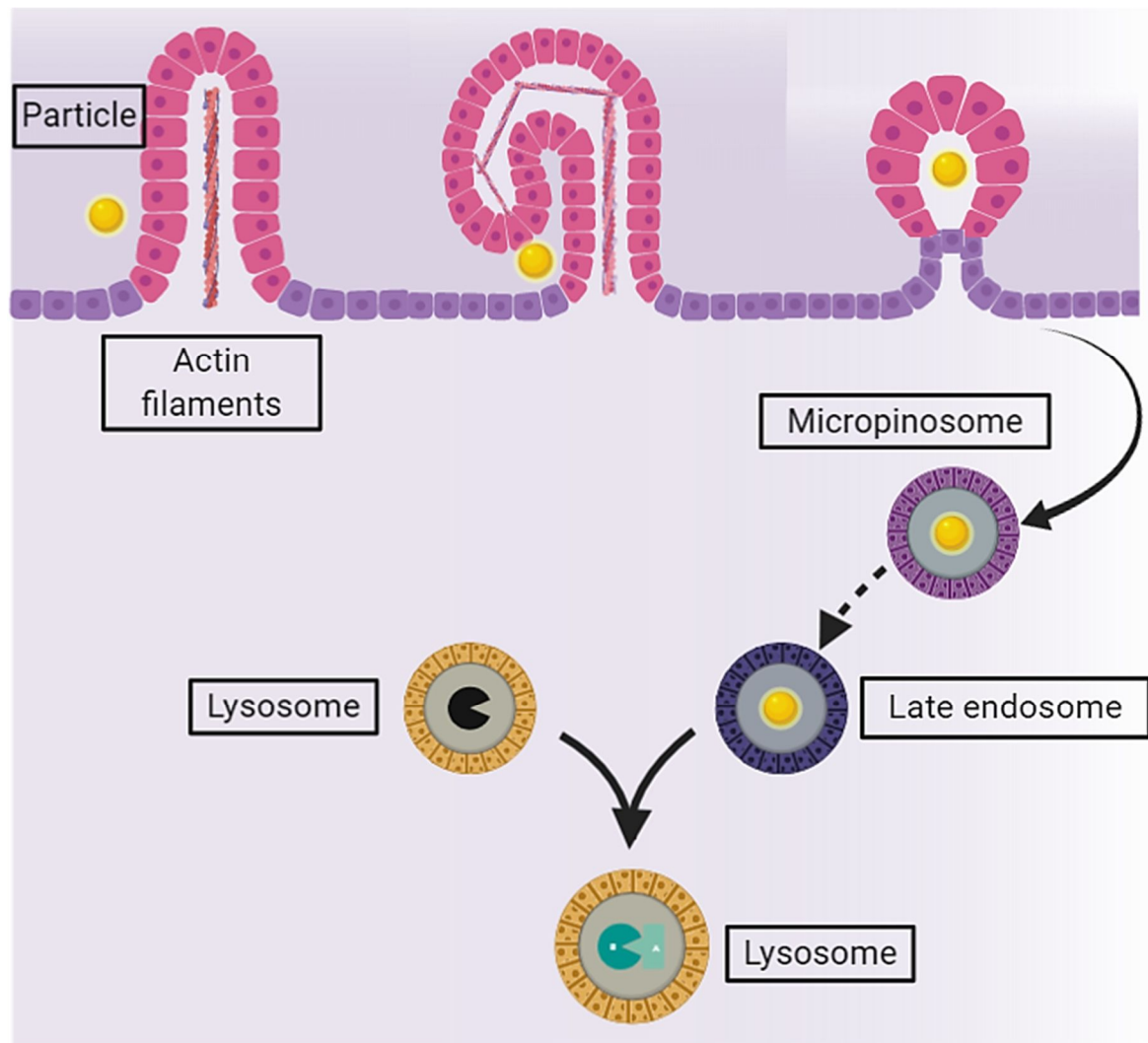


Figure 1.10: The process of micropinocytosis starts with an invagination of the cell membrane through the build-up of actin filaments. This creates a micropinosome which makes its way to the cell's cytosol maturing to the late endosome. Afterwards the Late endosome fuses with a lysosome containing digestive enzymes and in turn breakdown its contents.

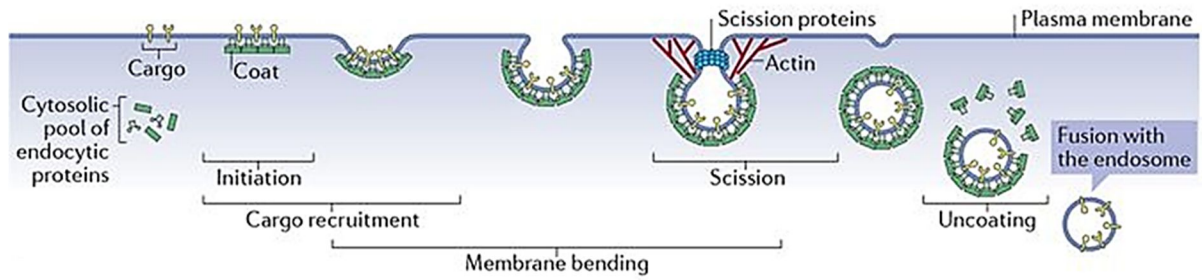


Figure 1.11: The process of receptor mediated endocytosis. This process starts with ligation of the antigen to the receptor which initiates binding of proteins creating a protein coat. This creates an invagination in the cell membrane where with the aid of actin and other proteins an endosome is formed. Once the endosome matures the protein coating gets recycled in the cell cytosol. adopted from [133]

1.2 Research objectives

Given their potent biological functions, carbohydrates are considered as promising tools for modulating different biological processes including immune responses. In fact, the immune modulatory effects of many naturally occurring carbohydrates (e.g. those expressed by pathogens or allergens), through engaging different CTLs expressed by dendritic cells, is well established. However, more research is required to understand the ability of monosaccharides in steering immune system towards pro or anti-inflammatory pathways with potential applications in different clinical settings including but not limited to vaccination, tissue regeneration, fighting infection and immunotherapy for allergy, cancer or autoimmune diseases. Such efforts have been hampered by a number of technical limitations. For example, given the heterogeneity of the naturally occurring polysaccharides they are often not very well characterised, and it is also challenging to provide the same structure in sufficient quantities necessary for functional experiments. This has resulted in the limited availability and reproducibility of carbohydrates which hinders functional and mechanistic studies as well as clinical applications [134].

In this project we aim to apply a high throughput screening strategy to investigate immune modulatory properties of a combinatorial library of synthetic monosaccharides both immobilised and in particulate form, as coatings on nanoparticles, with a particular focus on the ability of different monosaccharides in modulating key functional properties of human DCs. We hypothesise that distinct combinations of different monosaccharides are able to promote generation of immune stimulatory or regulatory DCs with the ability to initiate or suppress downstream T cell responses. Identifying such monosaccharides will not only provide novel tools for developing new strategies for immune modulation but also provide new insight into the immune modulatory role of monosaccharides and its molecular basis. We also hypothesise

that distinct carbohydrate combinations are able to interact with surface lectins enhancing nanoparticle uptake by DCs and their targeted delivery to specific intracellular compartments. Identifying these carbohydrate combinations will provide new methods for optimizing drug delivery, facilitating anti-cancer vaccinations and cellular activation towards adaptive immune responses.

Chapter 2:

Materials & Methods

2.1 Generation of human DCs and macrophages from peripheral blood monocytes

Whole blood was obtained from healthy individuals in accordance to the guidelines of the ethics committee and their approval (National blood bank, Sheffield, U.K.). Peripheral blood monocytes (PBMCs) separation was done by means of gradient centrifugation using Histopaque (Sigma-Aldrich, UK). To obtain CD14 monocytes, positive selection with magnetic beads using the cell separation system (Miltenyi Biotech, UK) was performed. Purity of cell separation was confirmed by flow cytometry. Following isolating, CD14 monocytes, were supplemented with complete RPMI media containing 10% heat inactivated Foetal Bovine Serum (FBS), 100µg/mL streptomycin, 100U/mL penicillin and 2mM L-glutamine (all from Sigma-Aldrich). Cells were then seeded (1×10^6 cell per well) in a 24 well tissue culture plate with the presence of 50ng/mL GM-CSF and 250U/mL IL-4 for dendritic cells or 50ng/mL M-CSF for macrophages (all from Miltenyi biotech). Cells are incubated at 37° in a 5% CO₂ humidified incubator for 6 days for differentiation where fresh supplemented media was added on day 3 [135].

2.2 Quantification of Indoleamine 2,3-dioxygenase (IDO) activity

Cells were treated as we have previously described. Cells incubated with the monosaccharide conditions were supplemented with L-tryptophan 100µM for the incubation period. Supernatant collected from the conditions were used to determine the IDO enzyme activity. This was done by measuring the levels of L-kynurenine in the supernatants using colorimetric method where 100µL supernatant are treated with 50µL of 30% trichloroacetic acid and centrifuged at 13,000 rpm for 5 minutes to precipitate proteins leaving behind metabolites (L-kynurenine). After centrifugation the mixture is collected leaving behind the pelleted proteins and 150µL of Ehrlich reagent is added [100mg of 4-dimethylamino benzaldehyde (Sigma) in

5ml of glacial acetic acid (Fisher Scientific)].[58] Using 384 well plates each 90ul of each condition is transferred to a well and the plate absorbance is determined using Promega GlowMax Explorer plate reader on a wavelength of 490nm. The results are plotted against L-kynurenine standards with concentrations ranging from 0-200 μ M.

2.3 Cell viability assays

2.3.1 Alamar Blue

AlamarBlue was used to determine cell viability (Thermo Fisher Scientific) in accordance to manufacturer's instructions. Cells were washed and seeded in 96 well plates with RPMI 1640 containing 10% AlamarBlue stain. Cells are incubated with dye for 4 hours and supernatant collected, and absorbance measured at 570nm using GloMax® plate reader from Promega.

2.3.2 Trypan blue

Trypan blue (Sigma Aldrich) was used to determine cell viability and presence of necrotic cells. A working solution of 0.04% was used to stain cells and analysis was carried out on Countess™ II FL Automated Cell Counter.

2.3.3 Fixable LIVE/DEAD

Cell viability analysis was done using the LIVE/DEAD Fixable Green Dead Cell Stain Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly cells were incubated with 1ul per mL of working solution for 20minuts at room temperature. Cells are then washed out of excess stain and fixed with 5% paraformaldehyde until analysis. Flow cytometry was carried out on FC500 (Beckman Coulter) and analysed using weasel software.

2.4 Flow cytometry

Cells were harvested and washed with PBA (PBS with 0.5% bovine serum albumin and 0.1% sodium azide). Incubation with labelled antibodies was then carried out in the dark at 4°C for

20 minutes. Afterwards Cells were washed in PBA and fixed with 4% paraformaldehyde until analysis. All samples are analysed using FC500 Flow cytometer (Beckman Coulter) and data analysis was carried out using Weasel software. Unstained was used to obtain MFI as illustrated in appendix Figure 1.

2.5 Cytokine quantification

2.5.1 Intracellular staining

Cells are fixed for 10 minutes using 5% paraformaldehyde and then resuspended and washed with 0.5 saponin (Biolegend) twice before staining. Cells are incubated with fluorochrome conjugated monoclonal antibodies IL10 PE-Cy7, IL12 PE-Cy5 and IL1b PE (all from Biolegend) for 30minutes. After incubation cells are washed with 0.5 saponin, resuspended and fixed with 5% paraformaldehyde until analysis. Data obtained from Astrios flow cytometer and analyzed using weasel software.

2.5.2 Sandwich ELISA

Cell-free culture supernatants were collected and stored at -80°C before analysis. The levels of cytokines IL-10, IL-12p70, IL-17, IL-4 and IFN γ were measured by sandwich ELISA using the Duo Set ELISA kit (R&D Systems, UK) according to manufacturer's instructions.

Briefly, 384 well plates were used where wells are incubated overnight with 4.00 μ g/mL capture antibody of the cytokine. After incubations wells are washed with wash buffer (0.05% Tween20 in PBS) in 3 cycles each time blotting sufficiently to avoid bubble formation. Subsequently, wells are incubated for 1 hour with reagent diluent (1% BSA in PBS filtered) the wells are washed in 3 cycles and samples /standards (ranging from 31.2-2000 pg/mL) are incubated for 2 hours in duplicates. Wells are washed and working dilution of Streptavidin-HRP (40 fold dilution) is added to each well and incubated for 20 minutes. Wells are washed and Substrate Solution (1:1 mixture of colour reagent A (H₂O₂) and colour reagent B

(Tetramethylbenzidine)) is added to each well. And incubated for 20 minutes. Finally stop solution (2 N H₂SO₄) is added and optical density of each well is analysed immediately, using a microplate reader set to 450 nm with correction set to 540nm. Samples were analysed using Glomax discoveries Promega plate reader and data analysed using Graphpad prism 7 to obtain concentrations from standard curve using the function Sigmoidal, 4PL, X is log(concentration).

2.6 T cell Isolation and co-culture

Autologous co-cultures were carried out with monosaccharides primed DC using T cells isolated from buffy coats. The process starts with isolating Pan T cells from buffy coat PBMCs using an immunomagnetic kit followed by naïve T cells separation using CD45RO immunomagnetic beads (both from Miltenyi Biotec).

DC-T cell co-cultured is carried out at a 1:10 DC to T cell ratio in RPMI1640 supplemented with 5% human AB serum 100µg/mL streptomycin, 100U/mL penicillin and 2mM L-glutamine (all from Sigma-Aldrich) On day 3 cells are supplemented with IL-2 2ng/ml and on day 6 cells pan T cells are ready for analysis while naïve T cells are restimulated with anti-cd3 and anti-cd28 overnight and are ready for analysis on day 7 [136].

2.7 Synthesis and Characterization of Polymer-Coated Gold Nanoparticles

Gold chloride trihydrate (HAuCl₄; > 49% Au, ACS grade) and thiol-terminated PEG (M_n = 3000 g/mol) were purchased from Sigma-Aldrich. Trisodium citrate (99.8%) was purchased from Acros Organics. Ultra-high-quality water with a resistance of 18.2 MΩ × cm² (at 25 °C) was obtained from a Millipore Milli-Q gradient machine fitted with a 0.22 µm filter. All other chemicals were used as received unless otherwise indicated.

Synthesis, characterization and coating of AuNPs was performed in Warwick University by our collaborators Dr. Matthew I, Gibson and Dr.Sarah-Jane Richards.

2.8 Synthesis of Citrate-Stabilized Gold Nanoparticles.

The synthesis of 16 nm diameter of gold nanoparticles is described. Briefly, 600 mL heated solution of a 0.85 mmol/L aqueous solution of HAuCl₄ to reflux in a scratch-free round bottomed flask. Then a ratio of 1:3 of Au/sodium citrate is made by adding 10.5 mL of a 0.5 mol/L of sodium citrate solution was added in a single portion to the HAuCl₄. The temperature was maintained at reflux for 30 min, during which time a deep-red coloration formed. The reaction mixture was then allowed to cool to room temperature over a period of 3 h. Assuming complete reduction of the HAuCl₄ into the particles, the total gold concentration in the final solution was 0.17 mg/mL [137].

2.9 Gold nanoparticle characterization

UV–visible absorbance spectra were obtained on a Varian Cary 100 Bio spectrophotometer operating at 25 °C using 10 mm path length cuvettes with a total volume of 2.5 mL. The method of Fernig and coworkers was used to estimate the diameter of citrate-stabilized gold nanoparticle using UV–visible spectroscopy [138].

Dynamic light scattering measurements were carried out on a Brookhaven Instruments system consisting of a BI-200SM goniometer and a BI-9000AT autocorrelator. A 100mW Ar⁺ ion laser (Lexel Lasers) operating at 488 nm was used. All measurements were performed at a temperature of 25.0 (±0.2) °C and at a scattering angle of 90°. Borosilicate cuvettes were used with a minimum of 3 mL of the nanoparticle solution. The nanoparticles were used at a concentration of ~0.05 mg/mL (total gold mass) in Milli-Q water and passed through a 0.4 µm filter before measurement [137].

2.10 Gold nanoparticles monosaccharide coating

Approximately 10 mg of the desired thiol-terminated amino-monosaccharide was dissolved in 0.5 mL of high-purity water with the pH adjusted to ~10 using 0.1 M NaCl solution (Fig.2.1).

Added to this tube, 120 mL of the citrate-stabilized gold nanoparticle solution (0.85 mmol/L total gold concentration) and then agitated overnight in the absence of light. To remove excess unbound monosaccharides, the particles were centrifuged for 3 h at 8000 rpm. Following careful decantation of the supernatant, the particles were then redispersed in 20 mL of high-quality water, and the centrifugation–resuspension process was repeated for a total of three cycles. After the final cycle, the particles were dispersed in 10 mL of high-quality water for future use. Assuming complete incorporation of the citrate-coated gold particles into the final polymer-coated particles, the total concentration of gold in the final solution was 10.2 mmol/L (2 mg/mL)

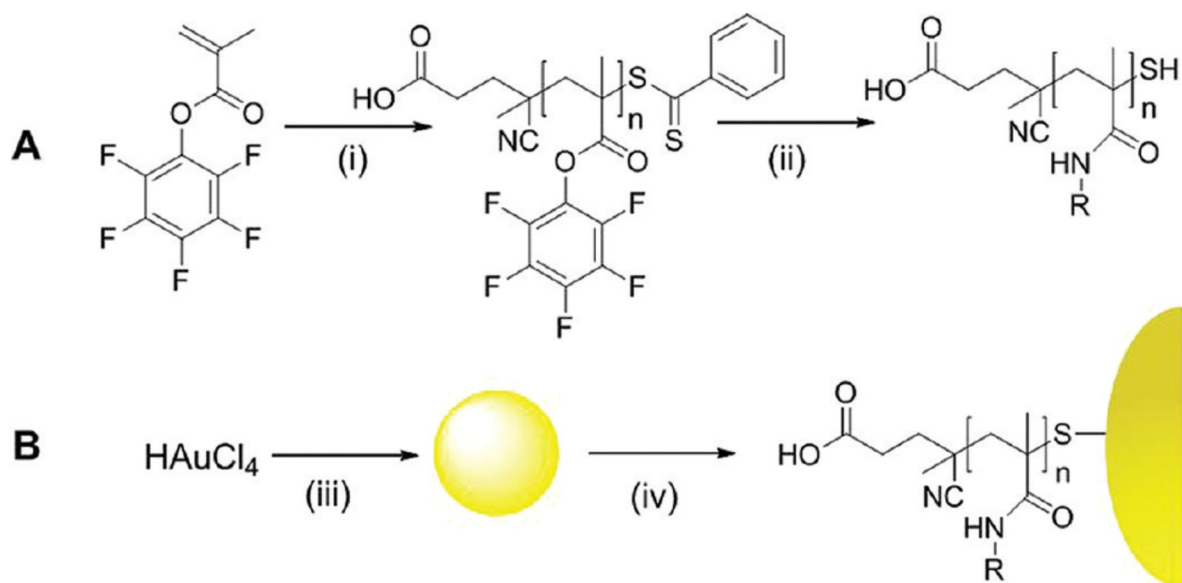


Figure 2.1: Illustrates functionalization of gold nanoparticles where A) is the substitution of pentafluorene with thiol B) Anchoring of the thiol to the gold nanoparticle. This figure was adopted from [139].

2.11 Preparation of AuNPs and cell culture

Gold nanoparticles were suspended by centrifugation with 8000rpm for 30 minutes. Excess water is aspirated, and the pallet is resuspended in RPMI 1640 supplemented with 10%FBS, 100µg/mL streptomycin, 100U/mL penicillin and 2mM L-glutamine (all from Sigma-Aldrich). 100ul of each of the resuspended gold particles are added to 400ul of supplemented medium containing 300k dendritic cells. Incubation is carried out in 37° in a 5% CO₂ humidified incubator overnight where they are analysed the following day.

2.12 Imaging flow cytometry

Conditioned dendritic cells and controls are stained intracellularly as described in chapter 2 for the assessment of particle uptake, lysosomes, endosomes and nucleus. Cells are fixed for 10 minutes using 5% paraformaldehyde and then resuspended and washed with 0.5 saponin (Biolegend) twice before staining. Cells are incubated with fluorochrome conjugated

monoclonal antibodies CD107a-PE-Vio615 (LAMP-1), CD71-PE-vio770 (Endosomes) (both from Biolegend) and DAPI (Sigma Aldrich) for 30minutes. AuNP conjugated with Hostasol green was used to look at uptake. After incubation cells are washed with 0.5 saponin, resuspended and fixed with 5% paraformaldehyde until analysis. Samples are analyzed using the MKII imagestream by Amnis and data is analyzed using IDEAS v6.2 Amnis software.

2.13 Statistical analysis

Statistical significance of the Data was obtained using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Therefore, paired t tests or ordinary one-way ANOVA was used when comparing between two groups. Statistically significant p values were considered to be equal or lower than 0.05.

Chapter 3:

Investigating the impact of immobilized monosaccharides on DCs phenotype and function

3.1 Introduction

Dendritic cells (DCs) are immune system's sentinels that are abundant in barrier tissues and sense their surrounding environment using a host of different pattern recognition receptors (PRRs) [140]. Upon activation DCs migrate to draining lymph nodes where they prime naïve T cells and instruct their differentiation towards different functional phenotypes (e.g. Th1, Th2, T-reg or Th17), a process that is heavily influenced by DC's surface phenotype, cytokine profile and expression of other immune-regulatory molecules [141]. DCs constantly sample their environment using PRRs, such as C-type lectins (CTLs), which enable them to transfer information about the nature of antigens and foreign materials they have encountered in peripheral tissues to T cells [142]. Different CTLs recognise different carbohydrates moieties and interaction between a given CTL and its ligand could influence DC phenotype and cytokine profile, and downstream events leading to T cell polarisation [143]. Carbohydrates are found on the surface of most cell types in nature and play key roles in recognition pathways and signal transduction. The wide range of functions carried out by carbohydrates is reflected in the structural diversity of carbohydrate molecules [144]. Carbohydrates can be monosaccharides, single carbohydrates such as mannose, galactose and fucose or polysaccharides, multi carbohydrates ranging from 2-20 linked such as mannans, galactans or fucans [85]. Some glycosylated proteins may comprise of up to 50% carbohydrates and loss of the carbohydrates group can result in loss of protein properties and functions leading to fatal consequences for a living cell [86].

Prime examples of these are the carbohydrate moieties decorating allergens and pathogen glycoproteins which modulates the immune response depending on their CTL counterpart. For example, it has been shown that binding of glycosylated allergens to the mannose receptor (MR) can induce a Th2 biased immune responses while same allergens could induce a Th1 response through binding to Dendritic Cell-Specific Intercellular adhesion molecule-3-

Grabbing Non-integrin (DC-SIGN) [87, 145]. Some intracellular pathogens are coated with mannose, such as *Mycobacterium* spp, promoting their uptake and survival inside host immune cells through inhibition of endosome-lysosome fusion [146].

DCs immune activity is also inferred in expression of immune regulatory enzymes such as 2,3 indoleamine dioxygenase (IDO). IDO is a rate-limiting enzyme that converts L-tryptophan into kynurenine, a metabolite with well-established immune modulatory properties. Our group have shown a link between IDO enzyme activity and CTLs activation, where allergens containing mannan interact with MR cause a reduction in IDO activity and promote a Th2 phenotype [58]. It is also worth mentioning that immune cells increase IDO activity during an infection causing an increase in kynurenine and depletion of tryptophan in the cell's microenvironment. This mechanism is used by immune cells to slow bacterial growth through decreasing tryptophan content in the microenvironment. This is also seen in tumours with high IDO activity depleting tryptophan and increasing immune regulatory kynurenine that suppress the immune cells and ultimately survival of the tumour [147].

Studies focusing on the anti-tumour activity of polysaccharides have shown promising outcomes for the development of novel anti-cancer therapies [148]. A study focused on the modification of a melanoma antigen 100gp with high mannose resulted in increased uptake of the antigen by DC-SIGN on DCs and increase antigen presentation to CD4 lymphocytes [103]. This enhanced presentation shows possible methods where carbohydrates are candidates for use as anti-tumour vaccines. Jia et al focused on plant based polysaccharide extracts from *Rhynchosia minima* in which exhibited anti-tumour activity *in vitro* against lung and liver cancer cell lines causing cell cycle arrest and apoptosis [149].

CTL distinguish self from nonself antigens through their pattern recognition mechanism enabling mounting of appropriate immune responses. DC-SIGN is known to be able to recognise mannose and fucose on glycosylated bacterial, fungal and viral antigens enabling

DCs to uptake and process these micro-organisms antigens and represent them to T cells to initiate the adaptive immune response [150, 151]. Some viruses such as HIV, relay on carbohydrates to bind to CTLs as a mode of transmission. Studies have shown that HIV binds Siglec1 through which it gains access to cervical DCs and then transferred to T cells leading to their infection and rendering them unable to mount an appropriate immune response [152, 153]. Different parasites have also been shown to utilise carbohydrates and CTLs to avoid the immune system. For example *Necator Americanus* has been shown to attract DCs towards its sheath, through a mechanism involving MR and DC-SIGN, allowing its infective larvae to escape unchallenged [154]. CTLs are also important when it comes to maintaining gastrointestinal homeostasis creating balance between inflammation and tolerance against gut microbiota such CTLs are the Dectins and Mincle [155, 156].

While much is known about how polysaccharides are able to modulate immune responses, particularly through changing DC phenotype and function, the ability of monosaccharides in modulating the immune system is not well studied. Nevertheless, the way the monosaccharide is presented to DCs (bound or soluble) plays a key role in guiding the immune response. This provides opportunities to utilize monosaccharides as immunomodulatory tools as anti or pro-inflammatory agents. In this context, this chapter investigates the effect of immobilized synthetic combinatorial libraries of carbohydrates in monosaccharide form consisting of mannose, galactose and fucose on DC phenotype, IDO enzyme activity, and cytokine production as primary readouts. Furthermore, the ability of monosaccharides primed DCs to skew T cell fate is investigated using DC-T cell co-cultures.

3.2 Methods

3.2.1 Isolated monocyte Purity test

Sample are prepared for flow cytometry on day 0 to assess CD14 purity and on day 6 to assess DC differentiation. In both cases samples are resuspended in PBS in FACS tubes and washed once. After that, the cell pellet is resuspended in PBA (PBS with 0.5% BSA and 0.1% NAN3) and centrifuged to discard the supernatant. Isotypes are used for each labelled antibody marker to check for unspecific binding that may occur against an unstained sample. Following incubation, the cells are washed to remove excess labelled antibodies and fixed with 0.5% paraformaldehyde (PFA) for later assessment or assessed directly using the FC500 flow cytometer (Beckman coulter) and data analysed using Weasel software.

3.2.2 Polymerization of *N*-hydroxyethylacrylamide

N-Hydroxyethyl acrylamide (HEA) (1 g, 8.69 mmol), pentafluorophenyl 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (PFP-DMP) (0.1844 g, 0.35 mmol), 4,4'-Azobis(4-cyanovaleric acid) (ACVA) (0.0195 g, 0.069 mmol) were dissolved in 50:50 toluene:methanol (4 mL). Mesitylene (150 μ L) was added as an internal reference. An aliquot was taken for NMR analysis in CDCl_3 . The solution was degassed under N_2 for 30 mins. The reaction was stirred at 70 $^\circ\text{C}$ for 90 mins. An aliquot was taken for NMR analysis in MeOD to determine conversion. The reaction was rapidly cooled in liquid nitrogen and precipitated into diethyl ether. The polymer was reprecipitated into diethyl ether from methanol twice to yield a yellow polymer product that was dried under vacuum. 97 % conversion by NMR, M_n (Theoretical) = 3400 $\text{g}\cdot\text{mol}^{-1}$ M_n (SEC) = 4100 $\text{g}\cdot\text{mol}^{-1}$ M_w/M_n (SEC) = 1.14.

3.2.3 Synthesis of 1-amino-1-deoxy-mannose, -galactose and -fucose

Galactose (mannose or fucose) (1 g) were stirred in 10 mL aqueous saturated ammonium carbonate for 2-5 days, ensuring the solution remained saturated. Reaction completion was determined by mass spectrometry. After reaction completion, the solution was freeze dried. Excess ammonium carbonate was removed by addition of warm methanol. After complete evolution of CO₂ the methanol was removed *in vacuo*. ESI-MS: 1-amino-1-deoxy-mannose (C₆H₁₃NO₅) M = 179.17 expected, [2M-H]⁻ observed 359.1 (C₁₂H₂₅N₂O₁₀), 1-amino-1-deoxy-mannose (C₆H₁₃NO₅) M = 179.17 expected, [2M-H]⁻ observed 359.1 (C₁₂H₂₅N₂O₁₀) and 1-amino-1-deoxy-fucose (C₆H₁₃NO₄) M = 163.17 expected.

3.2.4 End group modification of PFP-polyN-hydroxyethylacrylamide using amino sugars

In a typical reaction, PFP-pHEA (100 mg, 0.029 mmol), 2-amino-2-deoxy-galactose (26 mg, 5 eq.) was dissolved in 2 mL DMF with a drop of TEA. The reaction was stirred at room temperature for 16 h. The polymer was precipitated into diethyl ether from methanol three times. The polymer was dialyzed against water using 1K MWCO tubing to remove excess unreacted amino-sugar and freeze dried to result in. IR indicated loss of C=O stretch corresponding to the PFP ester and ¹⁹F NMR confirmed the loss of the PFP group. This was carried out with 1-amino-1-deoxy-β-D-galactose, (Gal1) 2-amino-2-deoxy-β-D-galactose (Gal2), 1-amino-1-deoxy-β-D-mannose (Man1), 2-amino-2-deoxy-β-D-mannose (Man2), 1-amino-1-deoxy-β-D-fucose, as well as ethanolamine as a non-monosaccharide terminated control illustrated in figure 3.1 [157, 158]. Monosaccharide attached at C1 which are Man1, Gal1 and Fuc1 are referred to here as isomer1 while those attached at C2 which are Man2, Gal2 and Fuc2 are isomer2.

3.2.5 Functionalization of 3D-Epoxy 96-well plates with monosaccharide - functionalized polymers

A 0.1 mg.mL⁻¹ solution of monosaccharide-functionalized PHEA was made up in milliQ water. Solutions of polymers were made up and 100 mL of each of these solutions was applied to a branched and spacers (50nm thickness) epoxy functionalized flat bottom 96-well polystyrene plates and allowed to react for 30 mins at room temperature. Each solution was made in triplicate. After 30 mins incubation, the solution was removed, and the well was washed with water three times to remove any unreacted polymer. To make combinatorial libraries of different monosaccharides a 'grafting to' approach was used as we have previously described [159-164] with minor adjustments. The method involves synthesizing a polymer to enable anchoring the monosaccharides of interest to the epoxide-functionalised polystyrene plate. Briefly, the polymer is engineered to have a carbohydrate capture terminus and an immobilization terminus. *N*-hydroxyethyl acrylamide [165] was polymerized to a degree of polymerization (DP) of 20 using reversible addition-fragmentation chain transfer (RAFT) polymerization mediated by a pentafluorophenol (PFP) α -terminated RAFT agent (Fig 3.1A). Monosaccharides (mannose, galactose, fucose) were captured onto this polymer scaffold using amino-monosaccharides by simple substitution of the terminal PFP unit. The monosaccharide-functionalized polymers were then immobilized onto 3D-epoxidey 96-well polystyrene plates using the ω -terminal thiol from the RAFT agent (Fig 3.1B) *via* a simple incubation and washing steps. Various combinations of monosaccharides were prepared, ranging from 100% of one monosaccharide (mannose, galactose or fucose) to 100% of another (mannose, galactose or fucose), in steps of 10% to make a total of 11 different wells in triplicate for each library. This post-polymerization route ensures that all the polymers have the same initial chain length distribution and therefore reduced the variability between the libraries produced but allows for versatile end-group functionalization to make simple libraries with differing carbohydrate densities.

3.2.6 Preparation of monosaccharide library plates for cell culture and screening of DC response

In this experiment 96 well plates were pre-coated with different Sugar concentrations (plate bound) in 10% increments with a maximum of two Sugars per condition (Fig.3.2). Each of the wells was sterilized with 70% ethanol and excess ethanol is washed with sterile PBS.

Immature dendritic cells were incubated in each well for 24hrs where each well was seeded with 100k DC in the presence of 10ng/ml E-coli LPS and supplemented with L-tryptophan 100 μ M. Controls were made on rows of the plates which have the HEA polymer and are not coated with sugars. LPS stimulated dendritic cells (positive control), none stimulated dendritic cells (negative control).

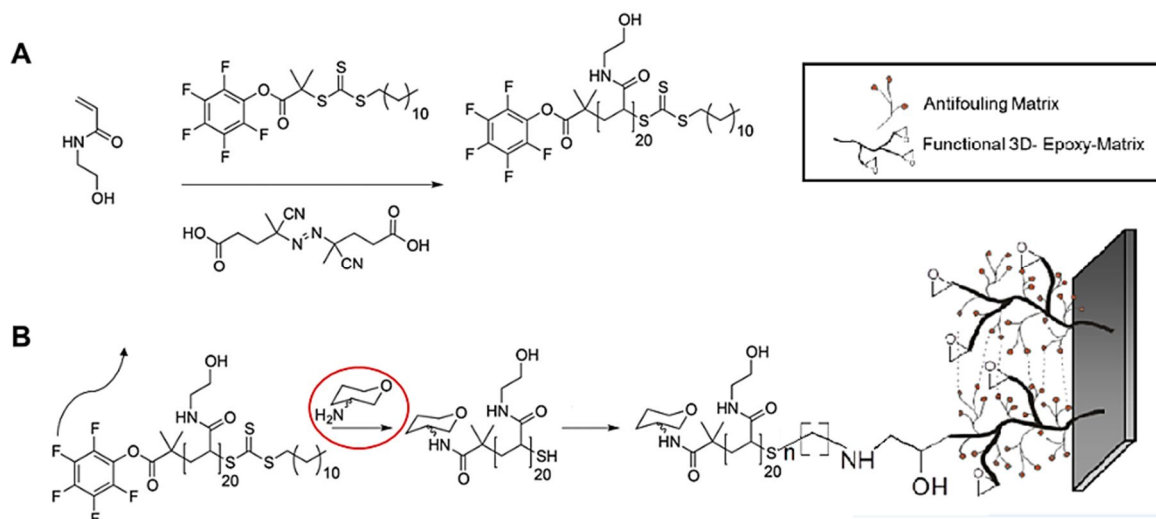


Figure 3.1: Explaining plate fabrication method used to coat plates with monosaccharides. Synthetic route to carbohydrate coated plates. (A) Polymerization of *N*-Hydroxyethyl acrylamide [165] *via* RAFT polymerization to a degree of 20. (B) Carbohydrate functionalization of PHEA and subsequent immobilization onto epoxide 96 well plates [166]

Plate 1													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	100 % Man-1	combinations of Man-1:Gal-1 in 10 % increments (3 repeats)									100 % Gal-1	unreacted epoxy	
B													
C													
D	100 % Man-2	combinations of Man-2:Gal-1 in 10 % increments (3 repeats)									100 % Gal-1		
E													
F													
G	negative control												
H	unreacted epoxy												

Plate 2													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	100 % Man-1	combinations of Man-1:Gal-2 in 10 % increments (3 repeats)									100 % Gal-2	unreacted epoxy	
B													
C													
D	100 % Man-2	combinations of Man-2:Gal-2 in 10 % increments (3 repeats)									100 % Gal-2		
E													
F													
G	negative contol												
H	unreacted epoxy												

Plate 3													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	100 % Man-1	combinations of Man-1:Man-2 in 10 % increments (3 repeats)									100 % Man-2	unreacted epoxy	
B													
C													
D	100 % Man-2	combinations of Gal-1:Gal-2 in 10 % increments (3 repeats)									100 % Gal-2		
E													
F													
G	negative control												
H	unreacted epoxy												

Plate 4													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	100 % Fuc-1	combinations of Fuc-1:Man-1 in 10 % increments (3 repeats)									100 % Man-1	unreacted epoxy	
B													
C													
D	100 % Fuc-1	combinations of Fuc-1:Gal-1 in 10 % increments (3 repeats)									100 % Gal-1		
E													
F													
G	negative control												
H	unreacted epoxy												

Figure 3.2: Maps of monosaccharide libraries used in plate formats. Each of the plates consists of a maximum of two monosaccharide combinations in each well. The combinations start with monosaccharide-1 at 100% and subsequent wells incorporate monosaccharide-2 in 10% increments reaching to 100% of that monosaccharide creating a unique combination of monosaccharide attached polymers.

3.2.7 Soluble monosaccharides mixing studies

In these experiments powder form glycans [mannose, galactose and fucose (all from Sigma Aldrich)] were directly dissolved in complete RPMI 1640 medium as a stock and kept in -80° to be used in the experiments. Each experiment consisted of the glycans incubated with 250k immature dendritic cells that are supplemented with 100uM of L-tryptophan. Soluble monosaccharide mixing studies were performed with a concentration 50ug/ml of monosaccharide with or without 10ng E-coli LPS. After 24 hours incubation, supernatant is collected for IDO activity measurement and cytokine profiling (Table.3.1).

Soluble monosaccharide combinations		
100%Mannose	100% Galactose	100%Fucose
90% Mannose 10%Galactose	90% Galactose 10% Fucose	90% Fucose 10%Mannose
80% Mannose 20% Galactose	80% Galactose 20% Fucose	80% Fucose 20% Mannose
70% Mannose 30% Galactose	70% Galactose 30% Fucose	70% Fucose 30% Mannose
60% Mannose 40% Galactose	60% Galactose 40% Fucose	60% Fucose 40% Mannose
50% Mannose 50% Galactose	50% Galactose 50% Fucose	50% Fucose 50% Mannose
40% Mannose 60% Galactose	40% Galactose 60% Fucose	40% Fucose 60% Mannose
30% Mannose 70% Galactose	30% Galactose 70% Fucose	30% Fucose 70% Mannose
20% Mannose 80% Galactose	20% Galactose 80% Fucose	20% Fucose 80% Mannose
10% Mannose 90% Galactose	10% Galactose 90% Fucose	10% Fucose 90% Mannose

Table 3.1: Soluble monosaccharide combinations used in mixing studies. Combinations of up to two monosaccharides in 10% increments from one monosaccharide to the other.

3.2.8 Quantification of Indoleamine 2,3-dioxygenase (IDO) activity

As described in chapter 2 (section 2.2).

3.2.9 IDO activity inhibition studies

For the inhibition of IDO enzyme activity 1-Methyl-DL-tryptophan was used (Sigma Aldrich). Cells were incubated with concentrations (0.004mM, 0.01mM, 0.1mM, 0.2mM, 0.4mM) in the presence of LPS for 24hrs. After incubations supernatant was collected to perform ELISA cytokine analysis and viability testing using alamar blue.

3.3 Results

3.3.1 DC generation and characterization

Immature DCs were generated as described in chapter 2 using peripheral blood monocytes that were isolated from peripheral blood mononuclear cells (PMBCs) with a >90% purity (Fig.3.3). Mature dendritic cells were generated through LPS stimulation of immature dendritic cells with 10ng/ml E-coli LPS and phenotypically examined. The phenotype of both immature and mature DCs were analysed using flow cytometry. CD14 and CD11c expression was used to confirm differentiation from monocytes to DCs, and DC-SIGN (CD209), MR (CD206) and stimulatory receptors (CD80, CD86, CD40, HLA-DR) were used as maturation markers. Results shown in Fig.3.4 indicate that the monocytes were successfully differentiated into immature dendritic cells at day 6 showing expression of CD11c, CD206, CD209 and no longer expressing CD14. Through LPS stimulation an upregulation on maturation markers CD80, CD86, CD40 and HLA-DR can be noted while there is a reduction in expression of CD206 and CD209 as expected.

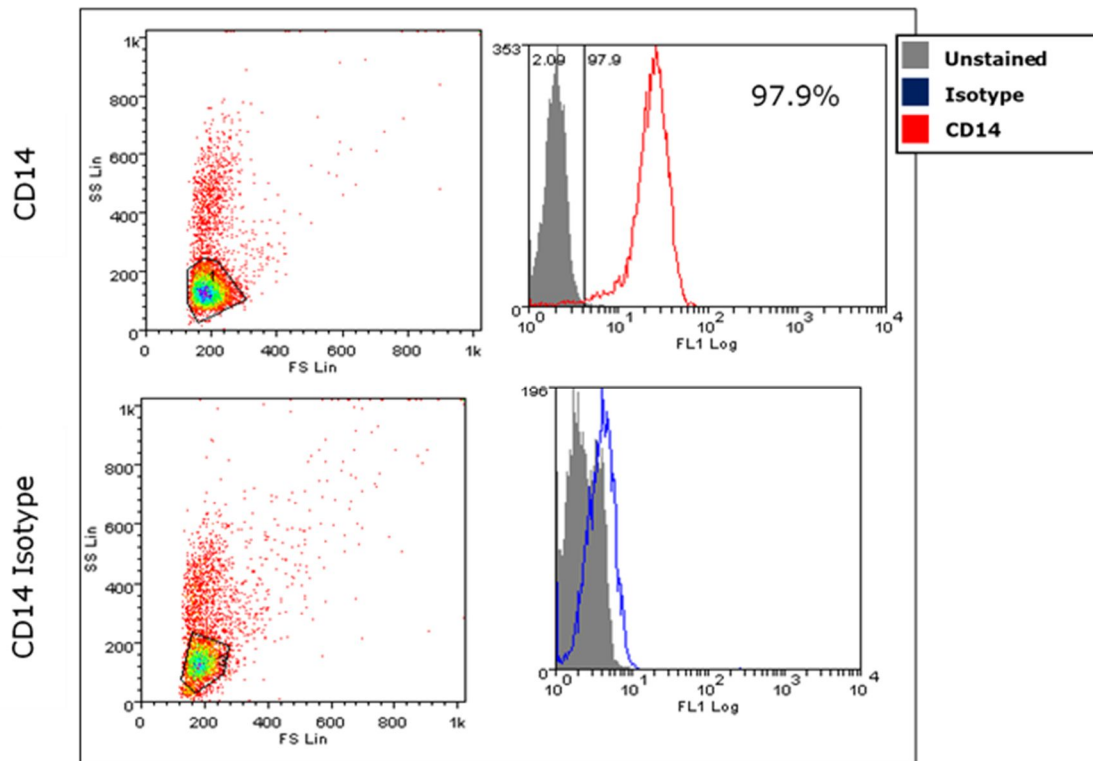


Figure 3.3: Flow cytometry staining of isolated monocytes from buffy coat. Data shows a purity of 97.9% CD14⁺ monocytes confirming the good quality of the isolation process and the yield of target cells. Data show results from a representative experiment of >6 independent experiments.

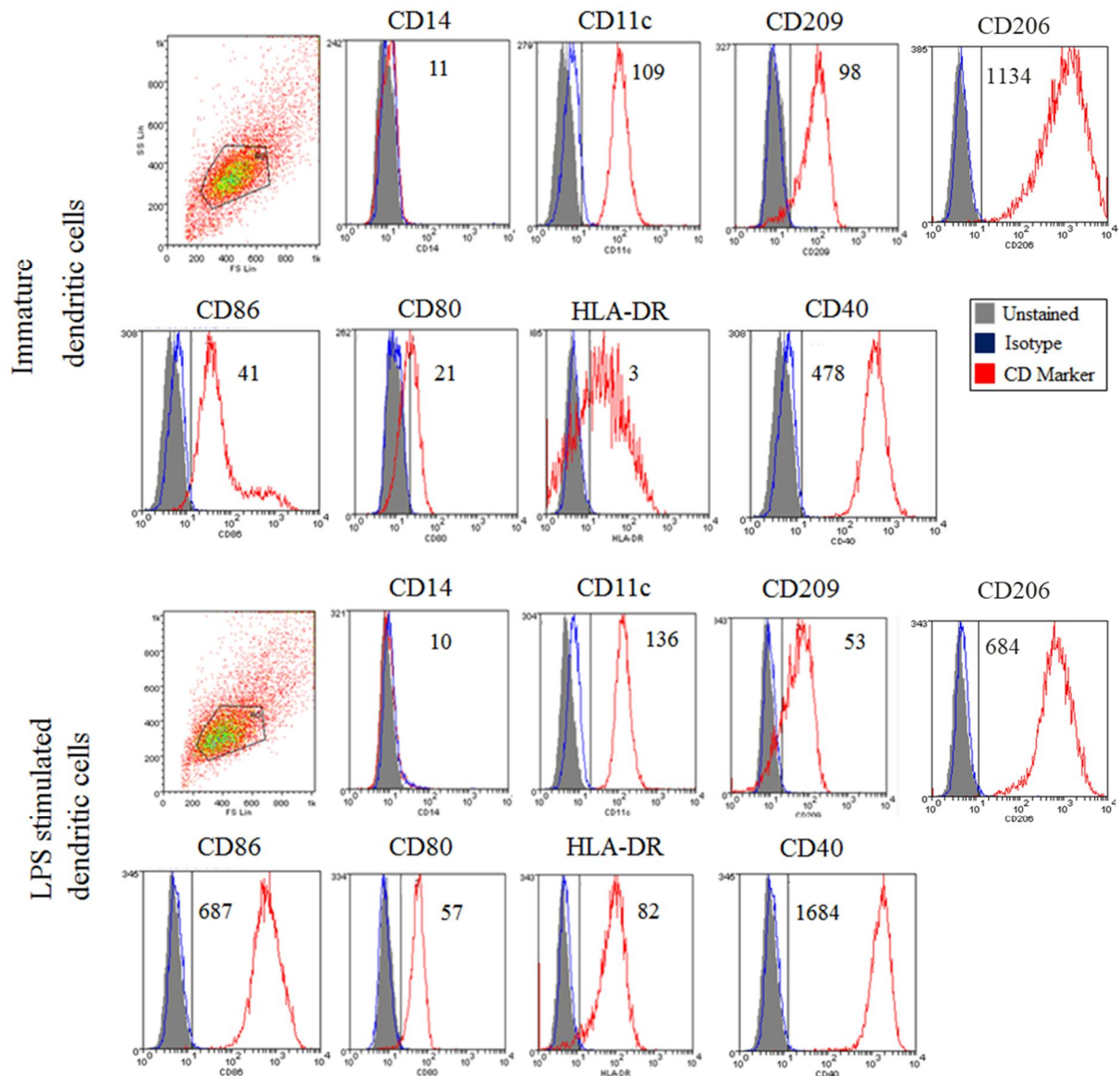


Figure 3.4: Flow cytometry phenotypic analysis of Immature and mature DCs. Immature dendritic cells generated show increased expression of CD209, CD206 CD11c, low expression of HLA-DR, CD80, CD86, CD40 and no expression of CD14 proving successful generation of immature dendritic cells on day 6. Following 10ng/ml E-coli LPS, stimulation expression of CD80, CD86, CD40 and HLA-DR increased while CD209, CD206 decreased and having maintained presence of CD11c with absence of CD14 show that dendritic cells were successfully matured. Data showing results in Median fluorescence intensity (MFI) from a representative experiment of 3 donors.

3.3.2 Effect of LPS stimulation on DC activation and viability

The importance of the cross talk between CTLs and TLRs in moulding the immune response has been evidenced by many studies both in allergy and immune responses against pathogens. It is well known that LPS ligation to TLRs induces cell activation and maturation in DCs guiding a pro-inflammatory response. This potent effect of LPS modulates IDO activity in DCs as shown by Nam et al. and is directly related to TLR-CTL cross talk as shown by Salazar et al. [58, 167]. In order to understand the immunomodulatory properties of carbohydrates on the TLR-CTL cross talk, optimum levels of LPS need to be utilized in which cell viability is not affected. To achieve this, we investigated dose response of LPS (*E. coli* O111:B4) on DCs with varying concentrations ranging from 0.001ng/ml to 100ng/ml. Looking at the data in Fig.3.5, concentrations from 0.001ng/ml to 1ng/ml induced little to no IDO activity while 10ng/ml and 100ng/ml achieved increased IDO activity in DCs. When considering cell viability, 100ng/ml had some toxic effects towards DCs while the 10ng/ml had no changes in cell viability while inducing IDO activity. From this data we decided to use the 10ng/ml of LPS to stimulate DCs and study the immobilized carbohydrates ability to modulate the TLR4-CTL cross talk and the downstream effects on the adaptive immune system.

Determination of IDO activity and cell viability relationship for LPS titration assay

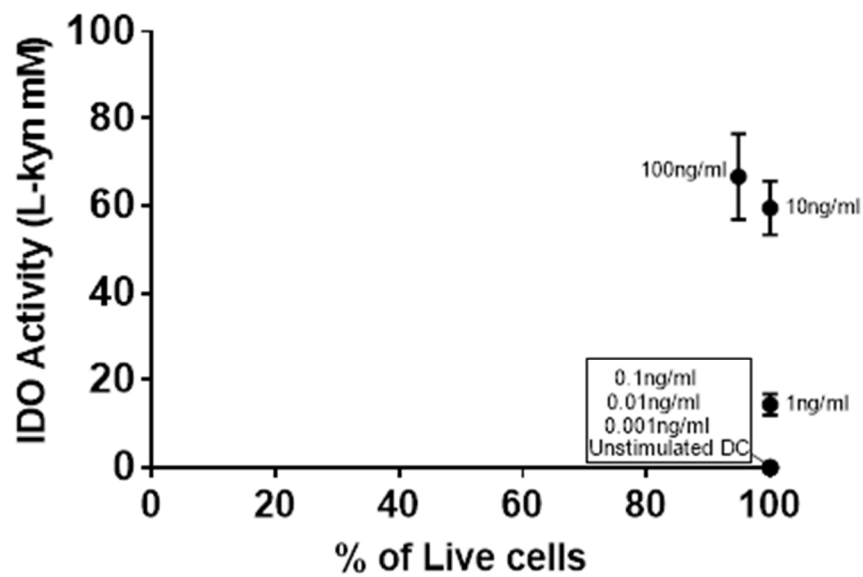


Figure 3.5: IDO enzyme activity relation to LPS concentration. The graph shows LPS titration assay for LPS concentrations (0.001, 0.01, 0.1, 10 and 100 ng/ml) where increased levels of IDO enzyme activity and minimal cell toxicity is obtained at 10ng/ml. Data from 3 independent experiments $\pm$ SD.

3.3.3 Immobilized monosaccharides modulate TLR4 activated DCs phenotype but not unstimulated DCs

To investigate the effect of monosaccharides on immature and mature DCs, unstimulated and LPS stimulated DCs were cultured on a library of monosaccharides consisting of different combinations of immobilized mannose, galactose and fucose (Table.3.2).

After 24hrs culture on immobilised monosaccharides, with or without LPS stimulation, flow cytometric analysis was carried out to look for phenotypic changes using surface markers and IDO activity was quantified using kynurenine in the supernatant as a surrogate for DCs immunogenicity. In this initial screen we focused on a selection of maturation and co-stimulatory receptors (CD86 and CD40), surface receptor linked to immune inhibition (CD274) as well as pro-inflammatory (IL-12p40 and IL-1 β) and anti-inflammatory (IL-10) cytokines. Data obtained show that in immature DCs (no LPS stimulation) monosaccharide combinations did not induce any changes in DCs phenotype, IDO activity or cytokine profile (Appendix Fig.2). However, flowcytometry data show that a selection of monosaccharides are able to modulate DC phenotype and cytokine profile in LPS stimulated DCs as evidenced by a significant decrease in CD40 expression, and an increase in PDL-1 expression suggesting a bias towards an anti-inflammatory phenotype in DCs stimulated under these conditions (Fig 3.6). This is also reflected in the cytokine profile where IL-10 is increased and IL-12p40 is decreased supporting the observed changes in the surface phenotype. This data clearly shows the immune-instructive potential of these immobilized monosaccharides.

Plate1	Plate2	Plate3	Plate4
100%Mannose1	100%Mannose1	100%Galactose1	100%Fucose1
90% Mannose1	90% Mannose1	90% Galactose1	90% Fucose1
10%Galactose1	10% Galactose2	10% Galactose2	10%Mannose1
80% Mannose1	80% Mannose1	80% Galactose1	80% Fucose1
20% Galactose1	20% Galactose2	20% Galactose2	20% Mannose1
70% Mannose1	70% Mannose1	70% Galactose1	70% Fucose1
30% Galactose1	30% Galactose2	30% Galactose2	30% Mannose1
60% Mannose1	60% Mannose1	60% Galactose1	60% Fucose1
40% Galactose1	40% Galactose2	40% Galactose2	40% Mannose1
50% Mannose1	50% Mannose1	50% Galactose1	50% Fucose1
50% Galactose1	50% Galactose2	50% Galactose2	50% Mannose1
40% Mannose1	40% Mannose1	40% Galactose1	40% Fucose1
60% Galactose1	60% Galactose2	60% Galactose2	60% Mannose1
30% Mannose1	30% Mannose1	30% Galactose1	30% Fucose1
70% Galactose1	70% Galactose2	70% Galactose2	70% Mannose1
20% Mannose1	20% Mannose1	20% Galactose1	20% Fucose1
80% Galactose1	80% Galactose2	80% Galactose2	80% Mannose1
10% Mannose1	10% Mannose1	10% Galactose1	10% Fucose1
90% Galactose1	90% Galactose2	90% Galactose2	90% Mannose1
100% Galactose1	100% Galactose2	100% Galactose2	100% Mannose1
100%Mannose2	100%Mannose2	100%Mannose1	100%Fucose1
90% Mannose2	90% Mannose2	90% Mannose1	90% Fucose1
10%Galactose1	10% Galactose2	10% Mannose2	10% Galactose1
80% Mannose2	80% Mannose2	80% Mannose1	80% Fucose1
20% Galactose1	20% Galactose2	20% Mannose2	20% Galactose1
70% Mannose2	70% Mannose2	70% Mannose1	70% Fucose1
30% Galactose1	30% Galactose2	30% Mannose2	30% Galactose1
60% Mannose2	60% Mannose2	60% Mannose1	60% Fucose1
40% Galactose1	40% Galactose2	40% Mannose2	40% Galactose1
50% Mannose2	50% Mannose2	50% Mannose1	50% Fucose1
50% Galactose1	50% Galactose2	50% Mannose2	50% Galactose1
40% Mannose2	40% Mannose2	40% Mannose1	40% Fucose1
60% Galactose1	60% Galactose2	60% Mannose2	60% Galactose1
30% Mannose2	30% Mannose2	30% Mannose1	30% Fucose1
70% Galactose1	70% Galactose2	70% Mannose2	70% Galactose1
20% Mannose2	20% Mannose2	20% Mannose1	20% Fucose1
80% Galactose1	80% Galactose2	80% Mannose2	80% Galactose1
10% Mannose2	10% Mannose2	10% Mannose1	10% Fucose1
90% Galactose1	90% Galactose2	90% Mannose2	90% Galactose1
100% Galactose1	100% Galactose2	100% Mannose2	100% Galactose1

Table 3.2: Immobilized monosaccharide libraries combinations. Each of the plates consists of a maximum of two monosaccharide combinations in each well. The combinations start with monosaccharide-1 at 100% and subsequent wells incorporate monosaccharide-2 in 10% increments reaching to 100% of that monosaccharide.

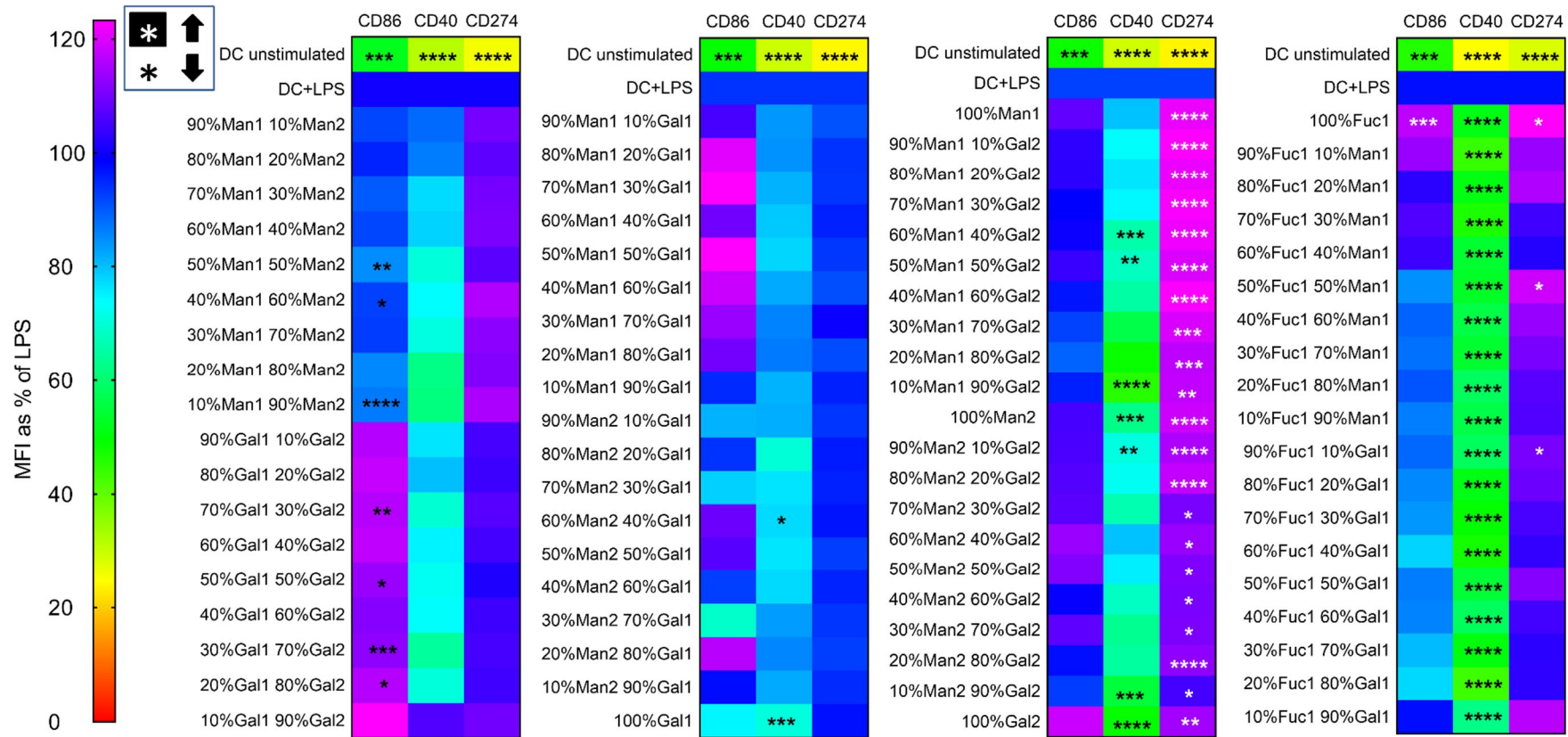


Figure 3.6: Flowcytometry screening of immobilized monosaccharides treated DCs phenotypic changes. Flow cytometry data shown as a heat map summarising expression profile of three surface markers (CD86, CD40 and CD274) on DCs after incubation on immobilized monosaccharide libraries in the presence of a TLR4 ligand (LPS). Unstimulated (immature) DCs were used as a negative control. All changes are expressed as a percentage of expression levels in mature (LPS stimulated) DCs. A reduction in CD40 expression was observed in most

conditions with highest reduction in fucose contained libraries compared to LPS alone. CD86 expression was higher in a number of conditions (particularly mixers of galactose1 and galactose2) however these did not reach statistical significance. CD274 (PD-L1) expression showed a significant increase in mannose1 and galactose2 combinations. Data is shown as mean $\pm$ SD of three independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3.4 Immobilized but not soluble monosaccharides modulate DCs IDO activity

Interaction between CTLs and their ligands depends on many factors that affect ligation and internalization of the glycosylated particles. Presentation of the glycosylated antigen or particle in soluble form or immobilized form would have different influences on the cell behaviour and ultimately cell response. To understand this in relation to carbohydrates immunomodulatory properties, DCs were presented with monosaccharides in soluble or immobilized forms followed by LPS stimulation. We hypothesised that monosaccharides in immobilised form have greater immunomodulatory effects than their soluble counterparts.

IDO activity assay for both soluble and immobilized forms was used as a tool to screen for changes in DC immunogenicity.

Data showed little to no changes in the IDO enzymatic activity in soluble monosaccharide treated DCs compared to LPS alone suggesting no significant influence on the TLR-CTL crosstalk (Fig.3.7). However, changes in IDO activity can be seen in DC treated with immobilized monosaccharides (Fig.3.8a-c). A selection of immobilized monosaccharides treated DC show significant reductions (Table.3.3) in the IDO activity more striking reductions in combinations mainly constituted of both isoforms of the monosaccharides where the monosaccharides are functionalized at C1 or C2 of the N-Hydroxyethyl acrylamide polymer for isomer1 or isomer2 respectively (see section 3.2.4). These findings on changes in both DCs phenotypic and IDO enzymatic activity are suggestive of immunomodulatory influence of the immobilized libraries and show the importance of the way the carbohydrates are presented to DC in shaping the immune response.

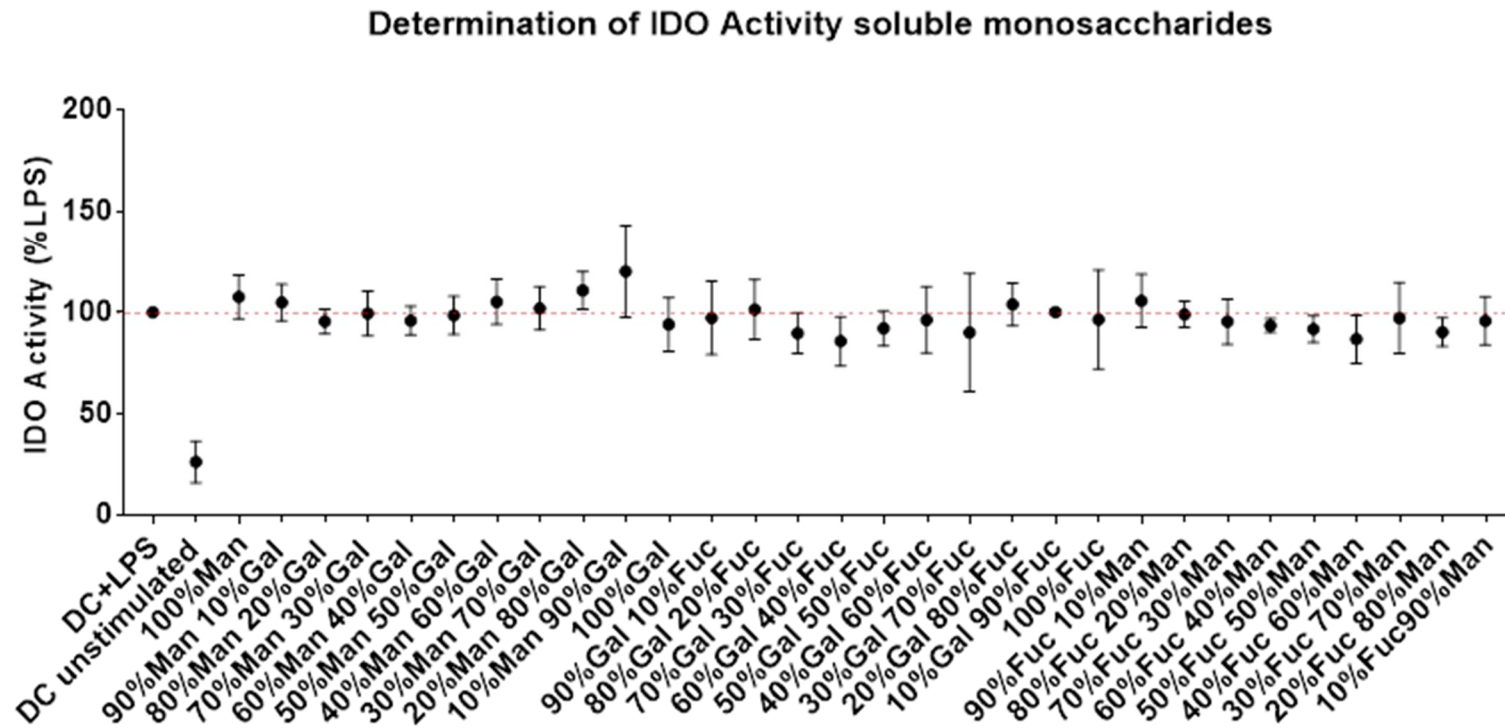


Figure 3.7: IDO enzymatic activity for soluble carbohydrate treated DCs (mixing studies). DCs were treated with 100ug/ml of a mixture of soluble monosaccharides and incubated for 24 hours to allow sufficient time for the CTLs to interact with the carbohydrates. No significance influences can be seen by the soluble form on DCs enzymatic activity and in turn on the cross talk between TLR4 and CTLs and the activity of the cells compared to LPS alone. Data is shown as mean $\pm$ SD of four independent donors.

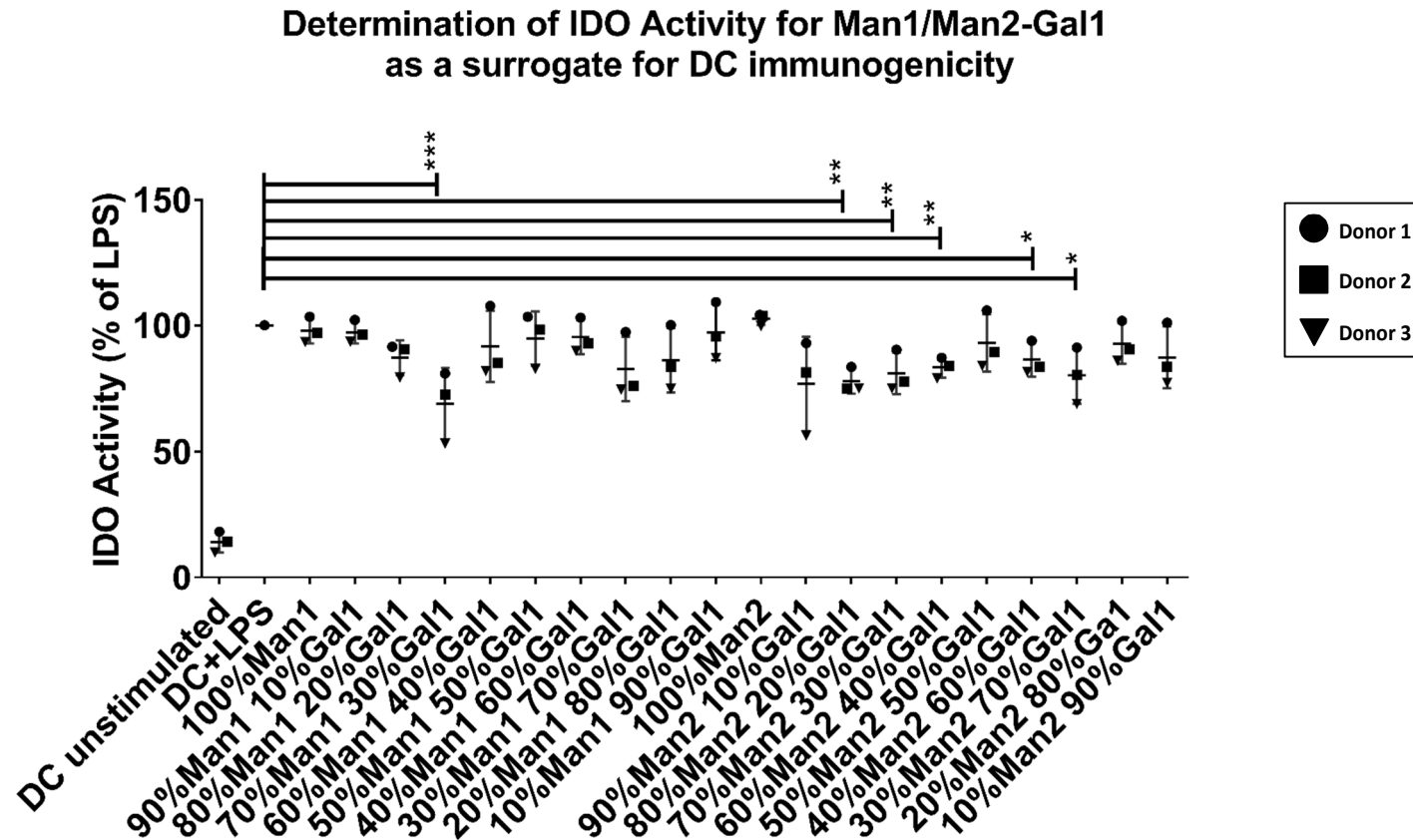


Figure 3.8a: IDO activity modulated in immobilized monosaccharide treated DCs for Man1/Man2-Gal1. Data obtained from IDO enzyme activity in DCs treated with the immobilized monosaccharides libraries in the presence of a TLR4 ligand (LPS). Reduction in IDO is prominent in monosaccharide combinations (70%Man1+30%Gal1, 80%Man2+20%Gal1, 70%Man2+30%Gal1, 60%Man2+40%Gal1, 40%Man2+60%Gal1 and 30%Man2+70%Gal1) while others have a reduction in IDO but not as significant as the later when compared to only LPS control. Data is shown as mean $\pm$ SD of three independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Determination of IDO Activity for Man1/Man2-Gal2 as a surrogate for DC immunogenicity

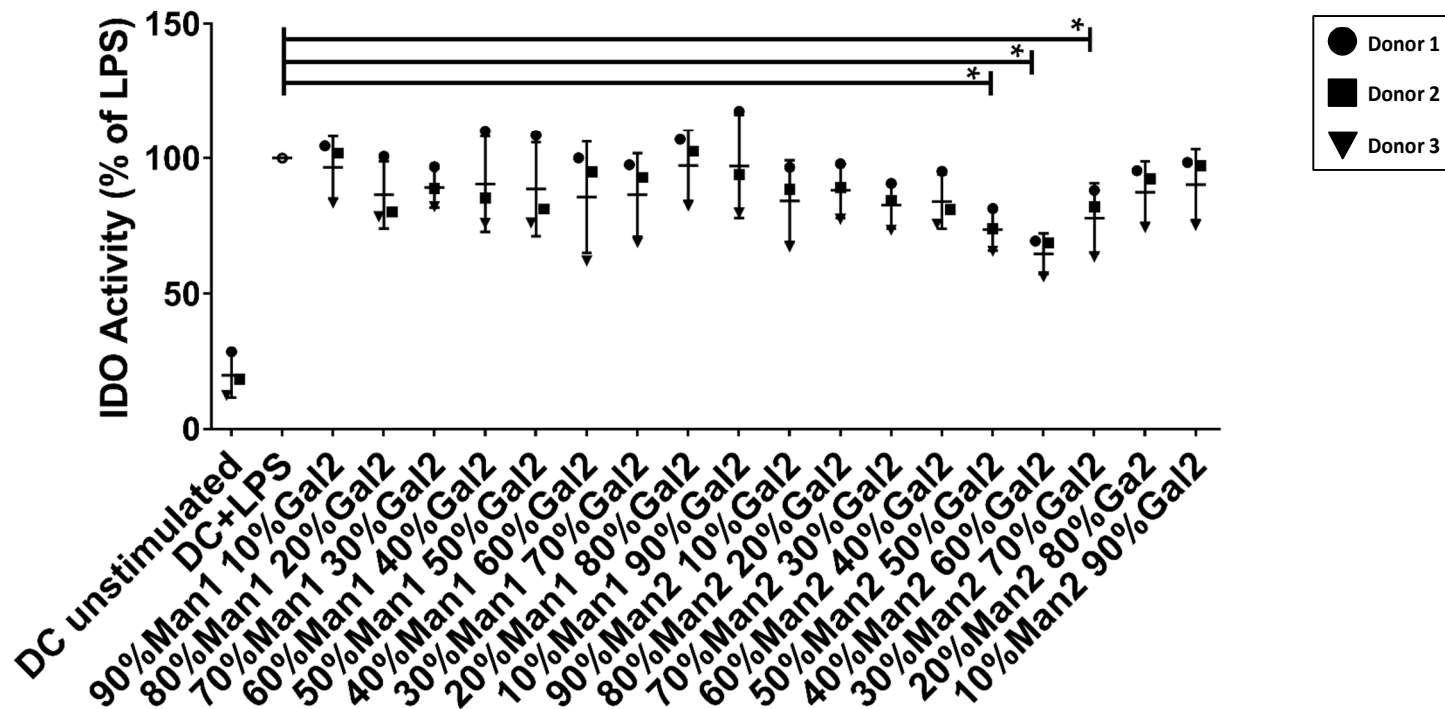


Figure 3.8b: IDO activity modulated in immobilized monosaccharide treated DCs Man1/Man2-Gal2. Data obtained from IDO enzyme activity in DCs treated with the immobilized monosaccharides libraries in the presence of a TLR4 ligand (LPS Reduction in IDO is prominent in monosaccharide combinations (50%Man2+50%Gal2, 40%Man2+60%Gal1 and 30%Man2+70%Gal1) while others have a reduction in IDO but not as significant as the later when compared to only LPS control. Data is shown as mean $\pm$ SD of three independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Determination of IDO Activity for Man1-Man2 & Gal1-Gal2 as a surrogate for DC immunogenicity

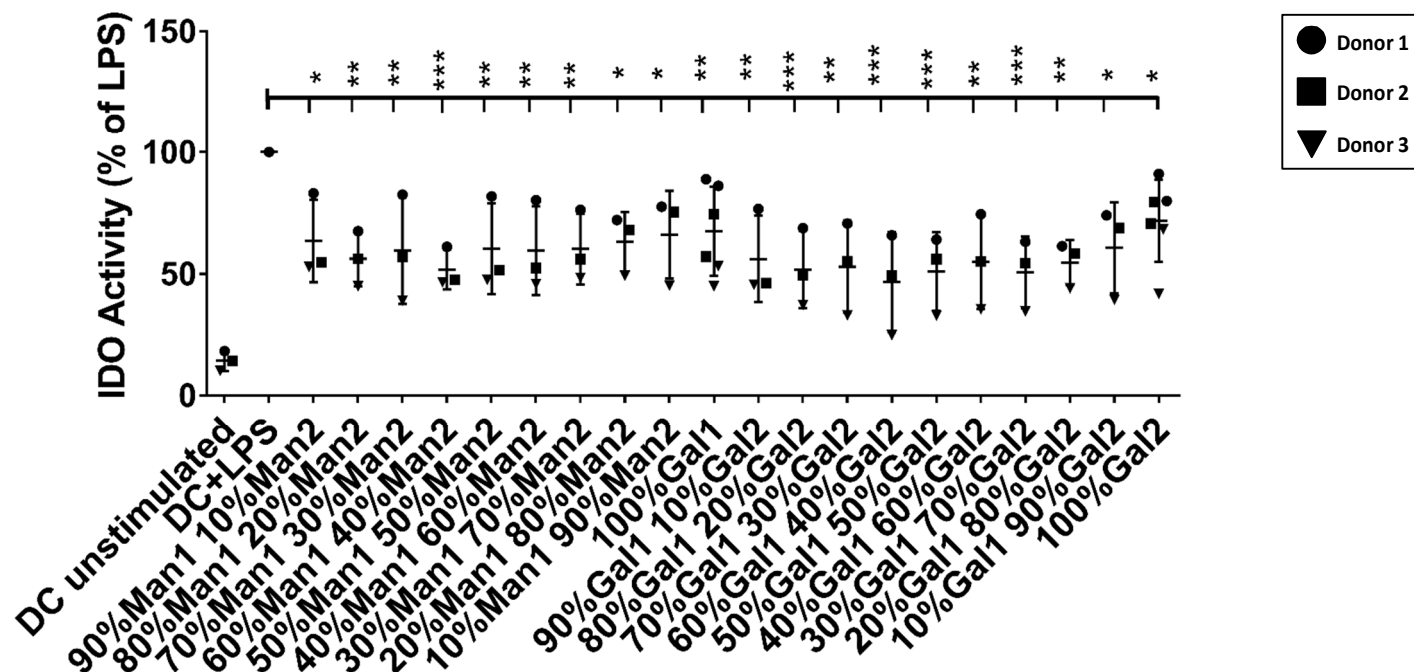


Figure 3.8c: IDO activity modulated in immobilized monosaccharide treated DCs Man1-Man2 and Gal1-Gal2. Data obtained from IDO enzyme activity in DCs treated with the immobilized monosaccharides libraries in the presence of a TLR4 ligand (LPS). Significant reduction in IDO is prominent in libraries with combinations consisting of the 2 isomers of the same monosaccharides when compared to only LPS control. Data is shown as mean $\pm$ SD of three independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

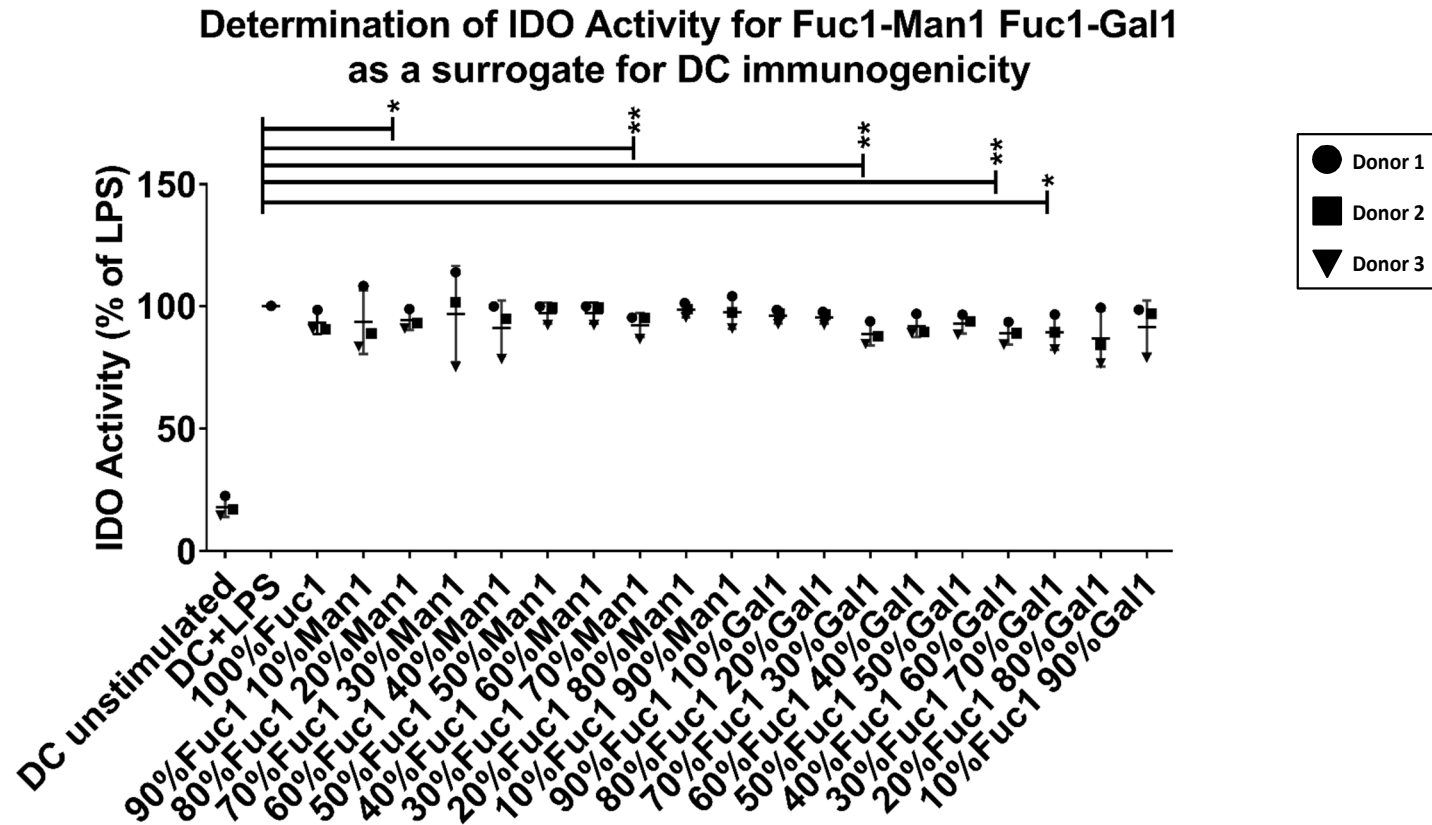


Figure 3.8d: IDO activity modulated in immobilized monosaccharide treated DCs Fuc1-Man1 and Fuc1-Gal1. Data obtained from IDO enzyme activity in DCs treated with the immobilized monosaccharides libraries in the presence of a TLR4 ligand (LPS). Reduction in IDO is prominent in monosaccharide combinations (80%Fuc1+20%Man1, 30%Fuc1+70%Man1, 70%Fuc1+30%Gal1, 40% Fuc1+60%Gal1 and 30% Fuc1+70%Gal1) while others have a reduction in IDO but not as significant as the later when compared to only LPS control. Data is shown as mean $\pm$ SD of three independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Condition	IDO modulation	Condition	IDO modulation
100% Galactose1	↓↓ IDO activity	90% Galactose1 10% Galactose2	↓ IDO activity
100% Galactose2	↓ IDO activity	80% Galactose1 20% Galactose2	↓↓ IDO activity
70% Mannose1 30% Galactose1	↓↓↓ IDO activity	70% Galactose1 30% Galactose2	↓↓ IDO activity
50% Mannose2 50% Galactose2	↓ IDO activity	60% Galactose1 40% Galactose2	↓↓↓ IDO activity
40% Mannose2 60% Galactose2	↓ IDO activity	50% Galactose1 50% Galactose2	↓↓ IDO activity
30% Mannose2 70% Galactose2	↓ IDO activity	40% Galactose1 60% Galactose2	↓↓ IDO activity
80% Mannose2 20% Galactose1	↓↓ IDO activity	30% Galactose1 70% Galactose2	↓↓ IDO activity
70% Mannose2 30% Galactose1	↓↓ IDO activity	20% Galactose1 80% Galactose2	↓ IDO activity
60% Mannose2 40% Galactose1	↓↓ IDO activity	10% Galactose1 90% Galactose2	↓ IDO activity
40% Mannose2 60% Galactose1	↓ IDO activity	90% Mannose1 10% Mannose2	↓↓ IDO activity
30% Mannose2 70% Galactose1	↓ IDO activity	80% Mannose1 20% Mannose2	↓↓↓ IDO activity
80% Fucose1 20% Mannose1	↓ IDO activity	70% Mannose1 30% Mannose2	↓↓ IDO activity
30% Fucose1 70% Mannose1	↓↓ IDO activity	60% Mannose1 40% Mannose2	↓↓↓ IDO activity
80% Fucose1 20% Galactose1	↓ IDO activity	50% Mannose1 50% Mannose2	↓↓↓ IDO activity
40% Fucose1 60% Galactose1	↓↓ IDO activity	40% Mannose1 60% Mannose2	↓↓ IDO activity
30% Fucose1 70% Galactose1	↓↓ IDO activity	30% Mannose1 70% Mannose2	↓↓↓ IDO activity
10% Mannose1 90% Mannose2	↓ IDO activity	20% Mannose1 80% Mannose2	↓↓ IDO activity

Table 3.3. Monosaccharide combinations that induced most significant changes in IDO activity. Significance of reduction in IDO activity is shown as ↓ $p < 0.05$, ↓↓ $p < 0.01$, ↓↓↓ $p < 0.001$.

3.3.5 Immobilized homologous isoforms, 100%galactoses and 90%galactose2 libraries modulate phenotype and IDO activity of TLR4 activated DCs

Initial phenotypic and IDO activity screening of the monosaccharide libraries have identified monosaccharides that have the highest immune modulatory effects (Table 3.3). These libraries consist of homologous isoforms, 100%galactoses and 90%galactose2 monosaccharides. These combinations were able to down regulate CD40 expression in LPS stimulated DCs which reached statistical significance. Furthermore, we observed an increase in CD274, the programmed death ligand 1 (PD-L1), expression on DCs treated with libraries of galactose2 indicating a shift towards regulatory DCs which enhances the survivability against T cells mediated attacks. No changes in CD86 expression were detected.

IDO enzymatic activity was determined using spectrophotometric detection of kynurenine after tryptophan supplementation to understand the carbohydrate libraries influence on DC TLR4 induced immunogenicity. Data obtained (Fig.3.8a-c) for show marked reductions in IDO activity where significant reductions can be seen in homologous isoform combinations whereas less reductions are seen in heterologous isoform combinations of monosaccharides. Interestingly this is amplified for 100% galactose1 as well as combinations including the galactose1. On the other hand, there are no significant reductions in 100%mannose1 and 100%fucose and much less reductions in mannose1 containing libraries. These observations show how monosaccharides are able to modulate DC enzymatic activity which is consistent with how pathogens manipulate the immune system into their favour.

3.3.6 Immobilized 100%Galactose1, 40%Mannose1+60%Mannose2, and 10%Mannose1,2+90%Galactose2 favor the production of TLR4-induced IL-10 in DCs.

Having investigated DC phenotype and IDO activity, the next step would be looking at the effect of different monosaccharides on DC cytokine profile. We choose to focus on IL-12 and IL-10 as prototypic pro and anti-inflammatory cytokines respectively. DCs stimulated with

LPS and cultured on 100%Galactose1 (Gal1), 40%Mannose1+60%Mannose2 (Man1-Man2), 10%Mannose1+90%Galactose2 (Man1-Gal2) and 10%Mannose2+90%Galactose2 (Man2-Gal2) coated well show striking increase in IL-10 while suppressing IL-12 production compared to LPS only (Fig.3.9a-c). These findings complement the observed changes in DC phenotype in the reductions of CD40 in these conditions and implies a regulatory phenotype in DCs treated with Gal1, Man1-Man2, Man1-Gal2 and Man2-Gal2. Moreover, significant reduction in IDO activity is in line with suppression of IL-12 in our conditions in example Gal1 and 50%Galactose1+50%Galactose2 (Gal1-Gal2). To follow up we decided to look at Gal1, Gal1-Gal2 as regulatory phenotypes inducers and Man1-Man2, Man1-Gal2 and Man2-Gal2 as pro-inflammatory phenotype inducers for our *in-vitro* co-culture experiments.

3.3.7 Immobilized 50%Galactose1+50%Galactose2, 60%Galactose1+40%Galactose2 reduce TLR4-induced IL-10, IL-12 and IDO activity in DCs.

An intriguing finding was identifying sugar combinations that were able to lower DC IDO activity as well as both IL-10 and IL-12 levels. Cells treated with 60%Gal1+40%Gal2 and 50%Gal1+50%Gal2 are believed to be leaning towards anergy given the low levels of enzyme activity and cytokine production. These conditions were also tested on macrophages to gain an understanding of the extent of influence these carbohydrates may have on lowering the innate APCs activities. We therefore looked at tumour necrosis factor α (TNF α) and IL-10 as well as IDO activity for macrophages treated with the selection of carbohydrates that caused anergy in DCs in the presence of a TLR ligand (LPS). Data obtained showed similar influence of the immobilized carbohydrates on macrophages lowering IDO activity as well as TNF α and IL-10 secretion in treated cells (Fig.3.10). These preliminary findings need more experiments to understand the potential of these combinations in modulating cells activity which would prove beneficial in combination with other chemotherapies to lower cancers dependant on IDO activity and reduce metastasis increasing overall successes of the cancer treatments.

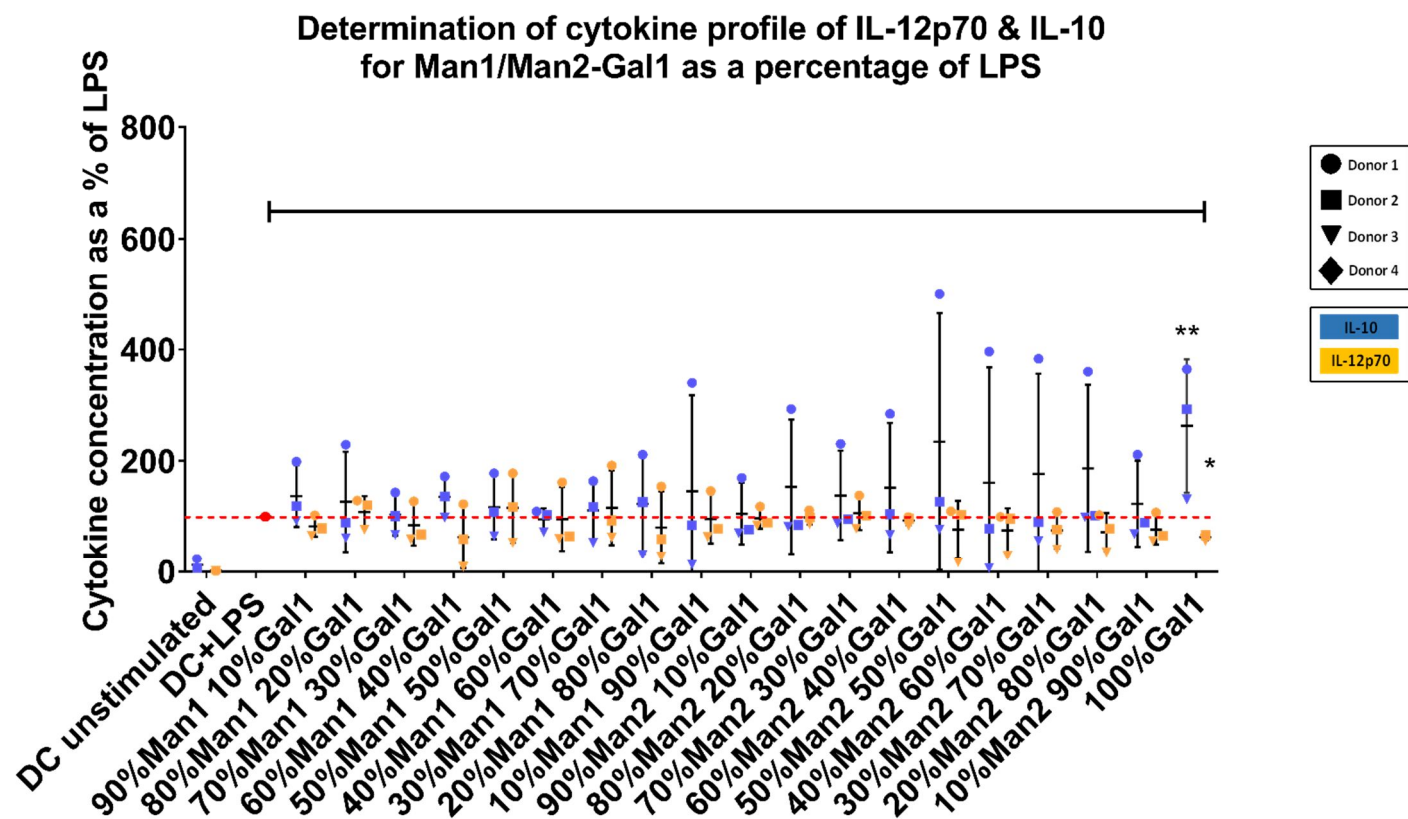


Figure 3.9a: ELISA data obtained for the cytokine profile (IL-10&IL-12) from supernatants of DCs treated with Man1/Man2-Gal1 in the presence of a TLR4 ligand (LPS). 100%Gal1 induce a significant increase in IL-10 production and suppression in IL-12 in response to LPS stimulation compared to LPS alone. Data are presented as the % change compared to levels of cytokines produced in response to LPS stimulation for each donor. Data is shown as mean $\pm$ SD of ≥ 3 independent donors where * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$, **** $p < 0.0001$**

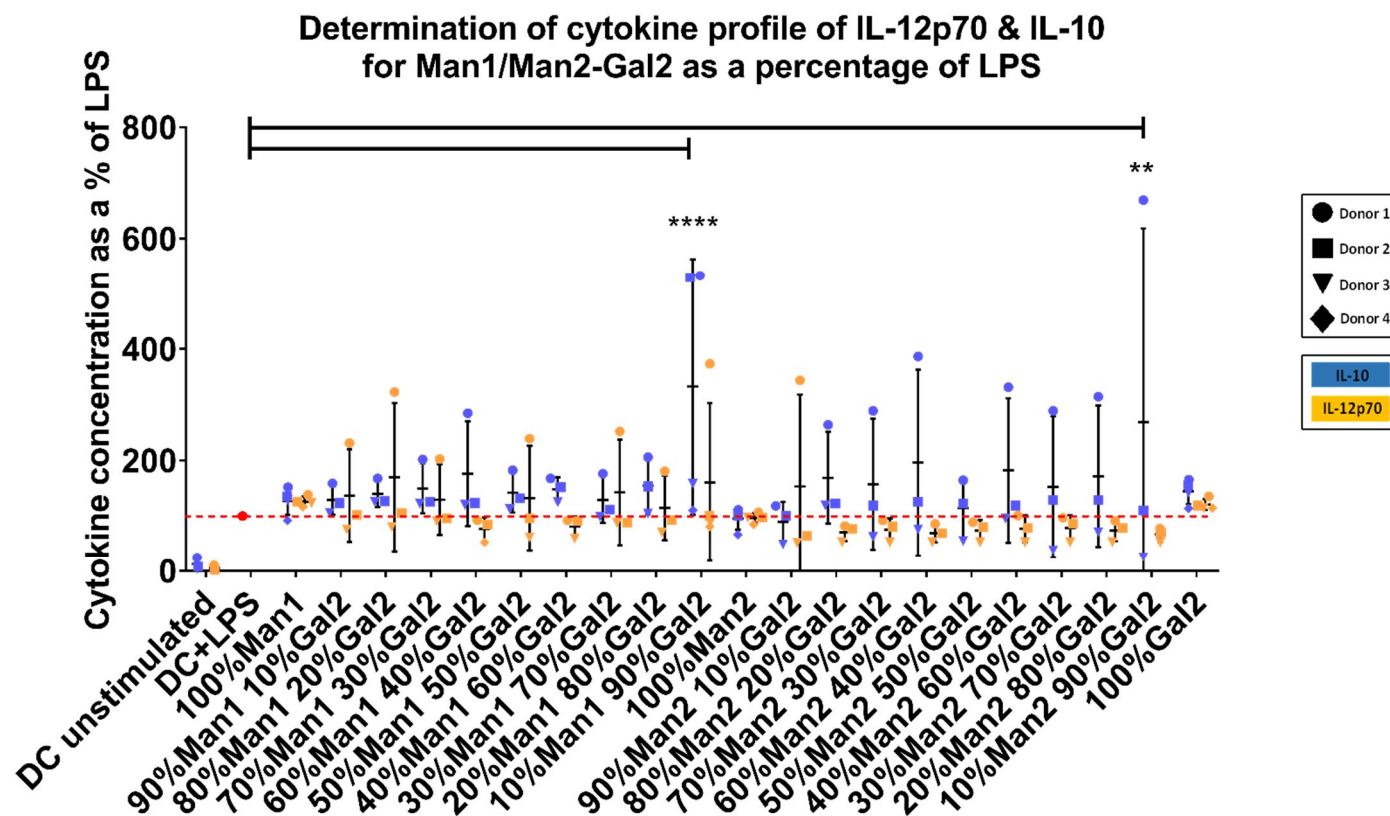


Figure 3.9b: ELISA data obtained for the cytokine profile (IL-10&IL-12) from supernatants of DCs treated with Man1/Man2-Gal2 in the presence of a TLR4 ligand (LPS). Monosaccharide combinations 10%Man1+Gal2 and 10%Man2+Gal2 induce a significant increase in IL-10 production compared to levels of cytokines produced in response to LPS stimulation for each donor. Data is shown as mean $\pm$ SD of ≥ 3 independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

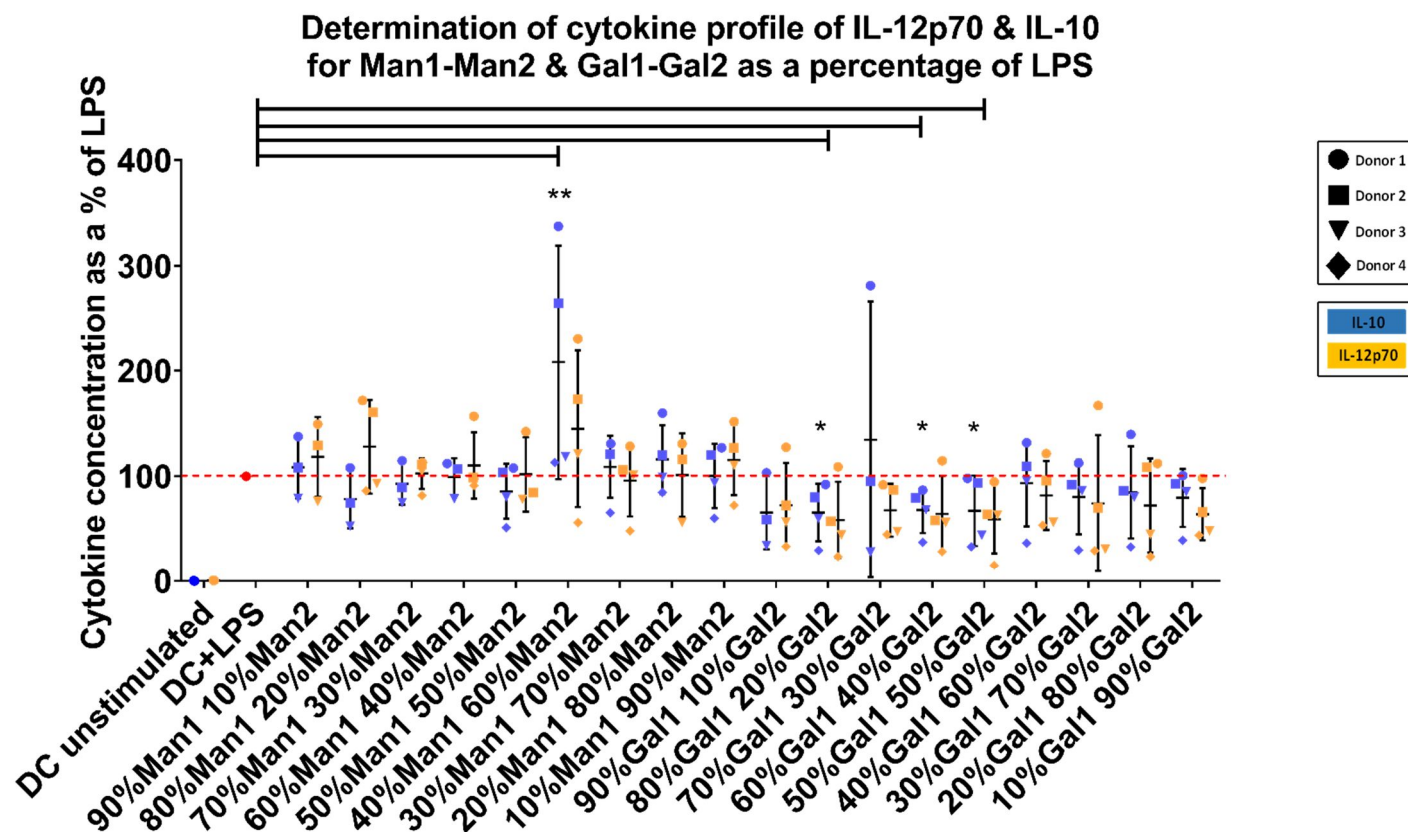


Figure 3.9c: ELISA data obtained for the cytokine profile (IL-10&IL-12) from supernatants of DCs treated with Man1-Man2 and Gal1-Gal2 in the presence of a TLR4 ligand (LPS). Combinations 40%Man1+60%Man2 induce a significant increase in IL-10 production while 80%Gal1+20%Gal2, 60%Gal1+40%Gal2 and 50%Gal1+50%Gal2 suppresses both cytokines in response to LPS stimulation most significantly suppressing IL-10 production compared to LPS alone. Data are presented as the % change compared to levels of cytokines produced in response to LPS stimulation for each donor. Data is shown as mean $\pm$ SD of ≥ 3 independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

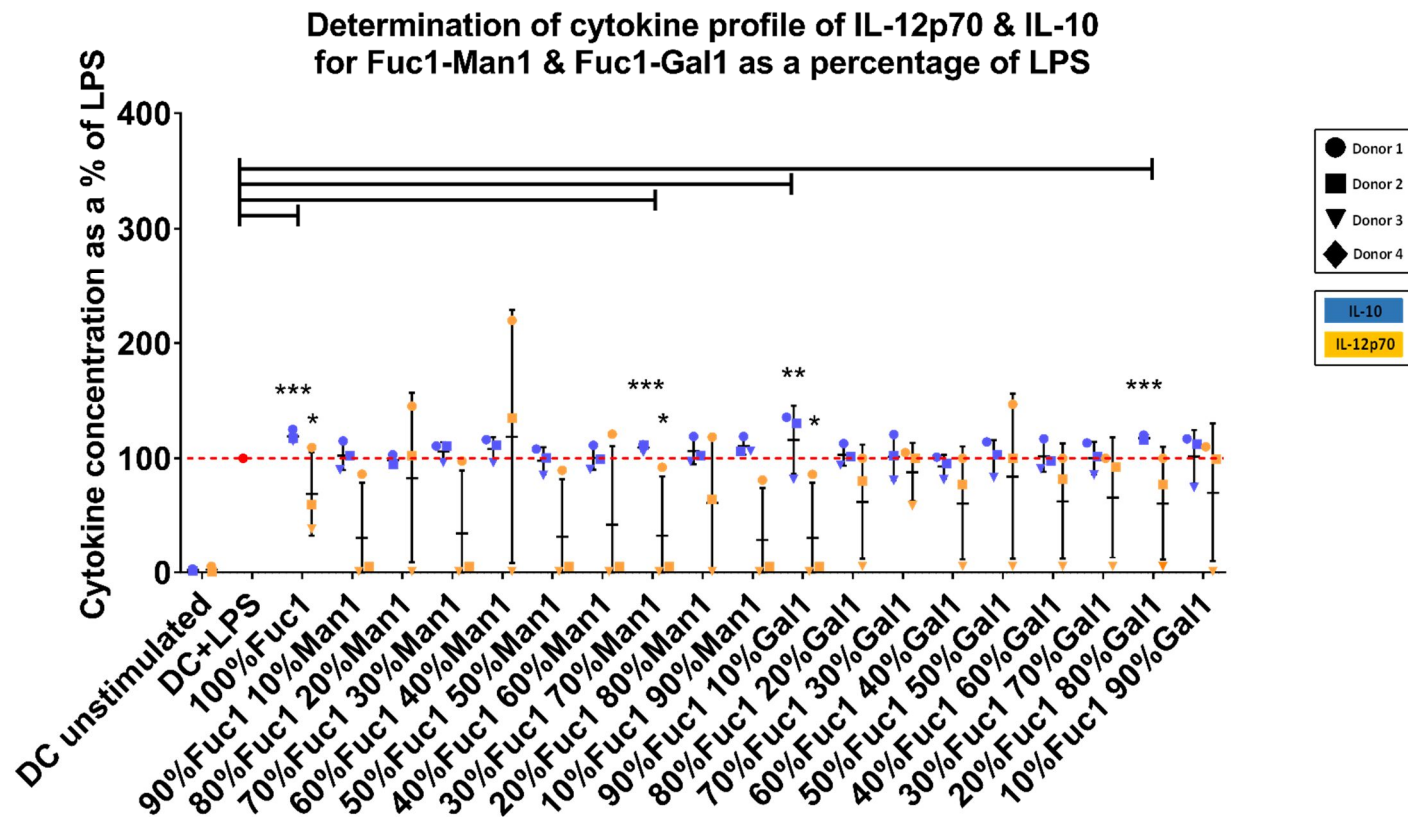


Figure 3.9d: ELISA data obtained for the cytokine profile (IL-10&IL-12) from supernatants of DCs treated with Fuc1-Man1 and Fuc1-Gal1 in the presence of a TLR4 ligand (LPS). 100%Fuc1, 30%Fuc1+70%Man1 and 90%Fuc1+10%Gal1 combinations induce a significant increase in IL-10 production and a significant suppression in IL-12 except for and 20%Fuc1+80%Gal1 which only showed an increase in IL-10 production compared to LPS alone. Data are presented as the % change compared to levels of cytokines produced in response to LPS stimulation for each donor. Data is shown as mean $\pm$ SD of ≥ 3 independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

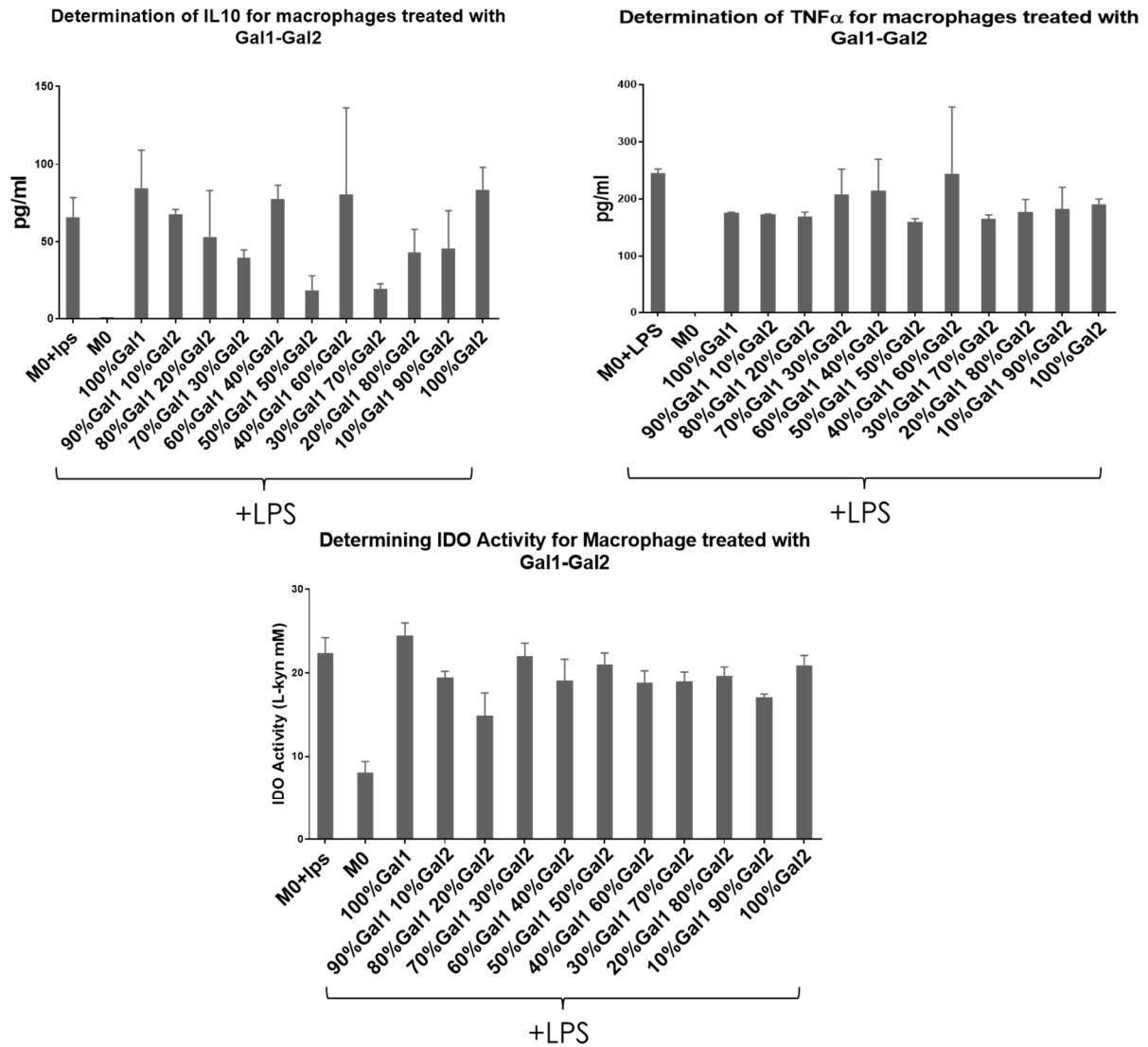


Figure 3.10: ELISA data obtained for the cytokine profile (IL10&TNFα) from supernatants of macrophages treated with a selection of immobilized carbohydrates in the presence of a TLR4 ligand. A decrease in IDO enzyme activity, IL10 as well as TNFα production is seen in most of the galactose combinations favouring present hypothesis in DCs where these conditions also caused lower cytokine secretion and enzymatic activity modulating TLR4 pathway via surface lectins compared to LPS alone. Data is shown as mean ± SD of ≥2 replicates of a single donor

3.3.8 The role of IDO activity in monosaccharide induced changes in DC cytokine profile

To investigate whether changes in IDO activity have a causal relationship with the observed changes in DCs cytokine profile we used 1-methyl-DL-tryptophan (MT), a competitive inhibitor of indoleamine 2,3 dioxygenase-1. Using this inhibitor, we successfully suppressed IDO activity in DC in a dose dependent manner with minimal toxicity reducing live cells by 4% and 7% at MT concentrations 0.2mM and 0.4mM respectively (Fig.3.11a,b). The cytokine profile for MT treated DCs show a reduction in the production of IL-12 compared to untreated DCs. This reduction is strongly correlated to the level IDO activity inhibited showing a direct correlation between IL-12 and IDO enzymatic activity. Interestingly, DC production of IL-10 is not significantly reduced until 0.2mM MT concentration where IDO activity falls below 10% compared to LPS only (Fig.3.11c). This is consistent with our findings where significant reductions in IDO activity seems to support significant increase in IL-10 but not for IL-12 production by DCs. We were able to show that pro-inflammatory effects are strongly correlated with an increase in IDO activity where a change in IDO is directly proportional to IL-12 production but not as strongly correlated to IL-10 production by DCs. Also, these effects have been compared to similar monosaccharides in soluble form where there was no reduction in IDO activity (Fig.3.7, 3.8a-c) which indicates the importance of mode of presentation of monosaccharides in obtaining optimum immunomodulatory potential.

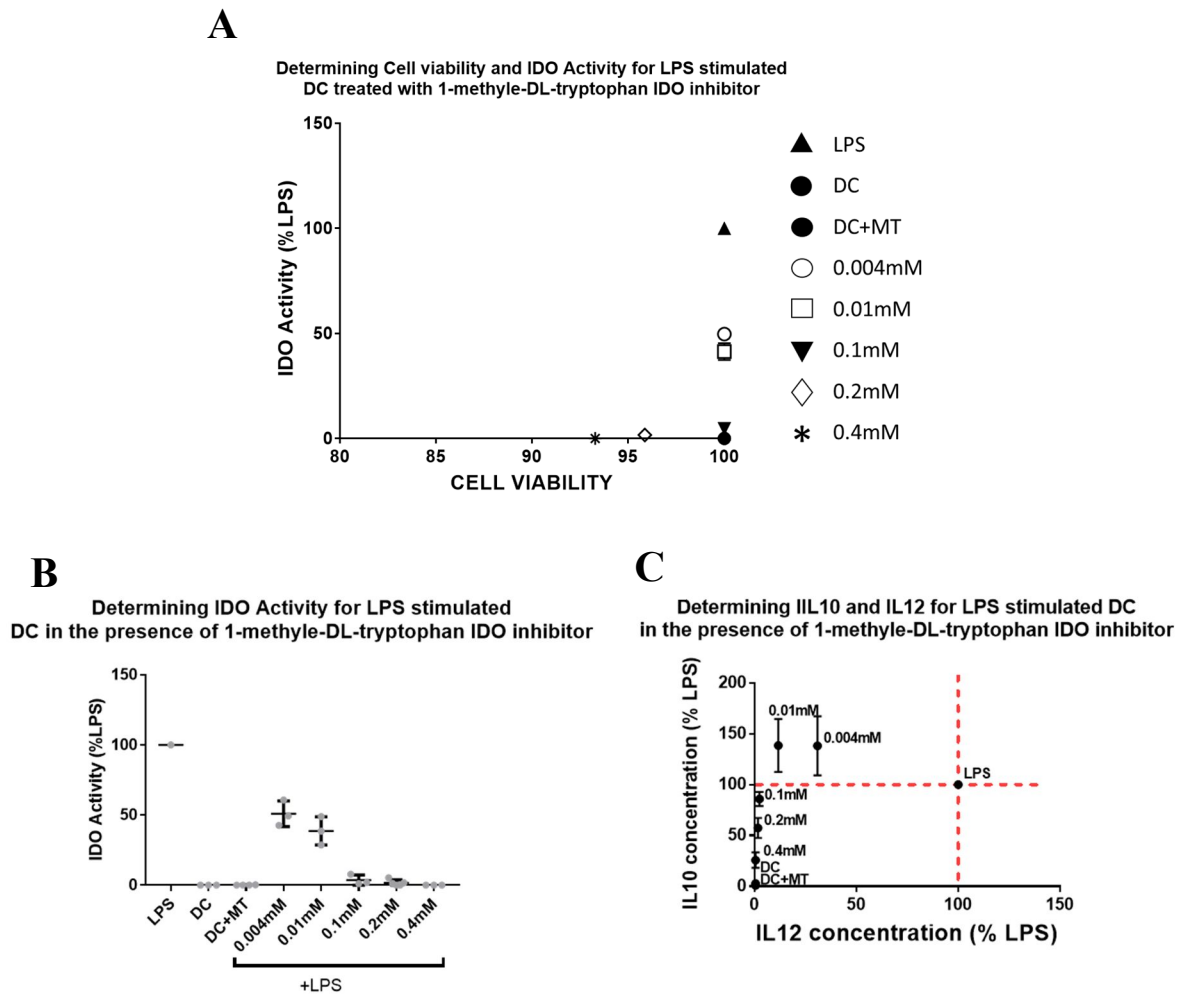


Figure 3.11: IDO inhibition studies for LPS stimulated DC using varying levels of 1-methyle-DL-tryptophan (a potent IDO inhibitor) IDO enzyme activity in relation to IL-10 and IL-12 production. A) IDO activity viability using alamar blue test showing minimal toxicity with reduction in live cells of 4% and 7% on concentrations 0.2mM and 0.4mM respectively. B) IDO activity was successfully inhibited using MT in a dose dependant manner. C) IL-12 has a proportional relationship to IDO enzyme activity while IL-10 production shows a peak in both (0.004mM and 0.01mM) where IDO is at 50% reduction and some IL-10 production in (0.4mM) which is 4 times optimum inhibition effect of MT on IDO enzyme activity. This indicates that IL-10 production is not completely reliant on IDO enzyme activity unlike IL-12 and regulatory phenotypes of DCs will be more prominent in IDO inhibition more than pro-inflammatory phenotypes. Data is shown as mean $\pm$ SD of 3 independent donors

3.4 Discussion

The central role of DCs in shaping adaptive immune responses makes them ideal targets for immunotherapy (e.g. anti-tumour therapies or treating autoimmune diseases) and vaccination [168, 169]. Carbohydrates decorating pathogens and parasites have been known to have an immunomodulatory effect on DC and the activation of the adaptive immune response. For example it has been shown that *Fasciola hepatica* glycoconjugates are able to modulate DCs by binding to DC-SIGN through the presence of mainly mannosylated terminal polysaccharides to promote anergy and regulatory phenotypes [170]. Also, Tn and sTn antigens containing galactose and N-acetylgalactosamine over expressed, exposed and aberrated on tumour cells [171]. These antigens are able to bind to MGL through their galactose end and cause suppression of DCs evading the immune system [172]. Another example is *H. pylori* LPS that is fucosylated and able to mimic human Lewis antigens interaction with dendritic cells with a suppressive outcome promoting the bacterial survival [173, 174]. On the other hand, mycobacterium LPS is mannosylated and is important for the organism's survival against the immune system by promoting inflammation and engulfment of the bacterium by attracted phagocytes where it is able to survive and cause disease [175]. Carbohydrates also play a role in allergen recognition and allergic sensitization by the immune system [176]. Allergens are glycosylated which hold a potential in interacting and activating the immune system the immune system. Reactions against allergens vary from mild itchy red spots to deadly anaphylactic shocks. Potency of the carbohydrates glycosylating allergens in modulating the immune response have been shown in literature [177]. This occurs through interactions between the carbohydrate moieties on allergens such as pollen through inhalation or peanut through ingestion with APCs. These cells once uptake allergens through surface lectins present antigens to T cells which in turn activate B cells to proliferate into plasma cells and produce IgE specific for the allergen in the sensitisation phase. Consequent exposures to the same

allergen would cause binding of the IgE to the allergen in which activates mast cells to degranulate producing its mediators (prostaglandins, histamines, leukotrienes). This causes the allergic reaction symptoms including swelling, hives, shortness of breath, gastrointestinal distress, nausea, vomiting etc. Studies have shown that monosaccharides glycosylating allergens are capable of binding to MR on DCs through their carbohydrate moieties to modulating the immune system [178, 179]. Another study shows that House dust mite allergens bind DC-SIGN through its carbohydrate moieties modulating the immune system [180]. More studies show the implications of mannose glycosylated allergens on the IDO activity of DCs where Der p1 a known ligand to MR is able to reduce DC IDO activity in HDM sensitive patients while increasing IDO activity in nonatopic patients [176, 181].

From a synthetic and translational perspective, using monosaccharides to modulate DC function is desirable as it significantly reduces the synthetic complexity associated with glycoconjugates, and by employing relatively simple mixing a high degree of diversity can be introduced. Furthermore, the use of immobilised monosaccharides provides advantages in yielding higher functionality and feasibility for coating different surfaces unlike their soluble counterparts. In this study we aimed to understand the immunomodulatory properties of monosaccharides on DCs using a combinatorial library of monosaccharides consisting of mannose, fucose and galactose.

Initial screens on immature DCs showed no phenotypic changes while TLR4 activated DCs showed modulation in CD40 and PD-L1 while maintaining CD86 expression. This indicates monosaccharide alone do not possess the potential to activate iDCs. Given the abundance of these monosaccharides in the human body it is not surprising that without accompanying a potent stimulus the immune cells would not react to them [182-184].

Looking at the data obtained for DC IDO activity (Fig.3.8), significant reductions in IDO activity are mainly seen in immobilized monosaccharide combinations of galactose and mannose isoforms while no IDO modulation is seen on their soluble counterpart. This is likely because DCs are not able to phagocytose the immobilized monosaccharides providing more opportunity for ligation to CTLs and stronger signalling depending on the type of monosaccharides-CTL interaction.

Galactose containing both isoforms Gal1 and Gal2 induced a significant decrease in LPS induced IDO enzyme activity and a general decrease in IL-10 and IL-12 where significant decrease in IL-10 can be seen in 50%Gal1+50%Gal2, 60%Gal1+40%Gal2 and 80%Gal1+20%Gal2. This alongside the decrease in CD40 shows a decrease in cells activity compared to unconditioned DCs. This interesting finding is similar to that of high IDO dependant tumours where inhibition of immune cells inflammation and increase regulatory phenotype is seen which leads to tumour survival. On the other hand, the decrease in IDO in Man1-Man2 conditions with no significance changes in IL-12 or IL-10 except for 40%Man1+60%Man2 where there is a significant increase in IL-10 is noted. Mannose is incorporated in glycoproteins of infectious organisms such as the glycoprotein R1016c of *Mycobacterium tuberculosis* and our findings of comparable levels of anti and pro inflammatory cytokine profiles suggests impairment of a Th1 phenotype and instead skewing of the immune response towards Th2 humoral response increasing susceptibility of the host to the disease [185, 186]. Mannose is also abundant in allergens such as HDM, Der p1, Canf and others where binding to lectins have shown an increase in IL-10 in atopic asymptomatic patients while the opposite have been seen in sensitive patients [181, 187]

IDO, is the rate limiting enzyme for the essential amino acid tryptophan catabolism and is known for its modulatory effects on immune cells and importantly in dendritic cells through more than one mechanism. The first known mechanisms is simply the depletion of the essential amino acid tryptophan from the micro environment [188, 189]. The second mechanisms is the accumulation of IDO activity by-products such as kynurenine causing feedback inhibition through the aryl-hydrocarbon [190-192]. Both mechanisms have shown to induce tolerogenic phenotypes of DCs which supports the survival of tumours and pathogens [193, 194]. Our data suggests significant decrease in IDO activity in a selection of immobilized monosaccharide treated DCs. The decrease in IDO activity was directly proportional to the decrease in inflammatory IL-12 but not to IL-10 cytokine production. These cells also exhibited reduction in costimulatory molecule CD40 expression which is involved in T cell activation [195]. Despite some contradictory evidence on the correlation between IDO activity and cell phenotype and function in the literature, it is clear that lack of IDO activity or reduced tryptophan levels in the microenvironment suppresses immune cells [196-198]. This is consistent with our findings where a reduction in IDO activity in DCs treated with a selection of immobilized monosaccharide corresponds to a tolerogenic phenotype as evidenced by reduction in CD40 expression and increase in IL-10 production.

Studies have shown that tumours bearing IDO having increased levels of IDO in response to cytokines from the environment lead to depletion of tryptophan. This causes other cells including DCs switching to anergic state and ultimately survival of the tumour cells [80, 199]. Carbohydrates coating microorganisms, parasites or even tumour cells seem to play a role in IDO modulation and thus pathogens' survival against immune cells [200]. Translating this into the context of infectious pathogens associated with expression of mannose as part of their glycoprotein shows how these pathogens can modulate the immune response into an anergic

state in many cases promoting the pathogens survival for longer periods against the immune system defence mechanisms. Understanding this would be very valuable in the development of anti-tumour therapies by modulating IDO and it goes along the lines of current tumour therapies where lowering IDO levels helps in eradicating tumours and make them susceptible to anti-tumour drugs and increase overall tumour therapy success in eradicating the cancer [80].

3.5 Conclusions

Data presented in this chapter show the immunomodulatory properties of a certain combinations of immobilized carbohydrates that are able to skew DC immune responses into inflammatory or anti-inflammatory phenotypes through modulation of the TLR-CTL cross talk. The most prominent ‘regulatory’ monosaccharides were Gal1 and Gal1-Gal2, and best performing pro-inflammatory sets were Man1-Man2, Man1-Gal2 and Man2-Gal2. Such combinations of monosaccharides can be used for therapeutic applications to promote anti- or pro-inflammatory immune responses as appropriate. Example applications include anti-tumour vaccine adjuvants for pro-inflammatory formulations or anti-inflammatory coatings on implantable medical devices to reduce inflammation for anti-inflammatory formulations. However, to fully understand the immune modulatory potential of ‘HIT’ monosaccharides, it is important to investigate downstream DC induced T cell activation and polarisation following priming DCs with different monosaccharide combinations. To this end co-culture experiments between monosaccharide primed DCs on T lymphocytes were carried out which is detailed in chapter 4.

Chapter 4:
Investigating the
immune-instructive
properties of DCs
primed on
immobilized
monosaccharides on T
cell polarisation

4.1 Introduction

Having shown the impact of immobilised monosaccharides on DCs phenotype, cytokine profile and IDO activity, it is important to understand the biological relevance of these monosaccharides ‘conditioned’ DCs in the context of DC induced T cell polarisation, the most important function of DCs. Therefore, in this chapter I wanted to investigate how monosaccharide conditioned dendritic cells instruct T cell polarisation which could shape the outcomes of the adaptive immune responses. Once DCs are activated and primed with antigens, they undergo maturation and migrate to lymph nodes where they interact with T cells [201]. It is well known that maturation and activation are linked intimately with the expression of costimulatory molecules and cytokine production in DCs [202]. Mature DCs possess the ability to present antigens and activate naïve T cells as well as memory T cells [203]. Depending on the activation stimuli, DCs exhibit different status translated in their surface marker expressions and cytokine profiles. DCs phenotype guide the activation and differentiation of naïve CD4⁺ T cells towards specialized unique populations of T cells. Effector T cells, once polarized by DCs, attain characteristics enabling them to fight infections and tumours or contribute to allergic disorders or autoimmune diseases under pathological conditions [204]. T helper cells differentiate into distinct functional phenotypes which can be broadly divided into anti-inflammatory (T-reg) or pro-inflammatory (Th1, Th2, Th9, Th17 and Tfh) subsets [205]. Each of the distinct population of T cells is suited to fight against certain types of infections. For example, Th1 are responsible for immunity against intracellular pathogens while Th2 are responsible for extracellular pathogens and is implicated in allergies [206]. DCs dictate the proliferation of T cells through the costimulatory molecules and cytokines secreted [207]. In an intracellular infection, IL-12 promotes Th1 differentiation while IL-10 inhibits the induction of this population and promotes T-reg generation [208]. The fine balance between anti and pro-inflammation can be

disrupted in conditions such as tumours and certain infections that have evolved to evade the immune system surveillance [209]. This is partially attributed to the carbohydrate content of the ligands that tumours and infections expose in interactions with the immune system specifically DCs [210, 211]. Studies have shown tumour fucosylated ligands that modulate DC-SIGN are able to evade the immune surveillance through suppression of DC functions and thus altering T cell activation [212]. Moreover, allergic immune responses have been attributed to carbohydrates ability in modulating CTLs responses and ultimately shift towards Th2 and away from Th1 [213]. Al-Ghouleh et al showed that glycosylated Der p1 is interacts with MR and deglycosylation of Der p1 leads to reduction in the uptake of the allergen. The reduced uptake of allergens through deglycosylation shows the importance of carbohydrates in the context of allergy TH2 generation and downstream IgE production.[135].

CTLs have also been shown to be a gateway to transfer HIV to T cells [214]. The virus has developed to have high mannose content on its envelope protein (gp120) leading to high specificity towards DC-SIGN [215].

T-cells subtypes may contribute to more than one function or shift from one subtype to another, in response to environmental cues, a term referred to as plasticity [216]. This occurs when changes in stimulus and microenvironment causes reprogramming of the cell's genetic material and transcription factors [217]. The reprogramming can enhance or suppress the original T cell phenotype. A recent study by Solomon et al. has shown that T-cells expressing FOXP3⁺ RORγt⁺, main transcription factors associated with T-regs and Th17 respectively, act as regulatory of inflammation even though the classic role of Th17 is pro-inflammatory [218, 219]. This occurs when a population of T-regs gets reprogrammed into expressing RORγt alongside the regulatory gene FOXP3.

Anti-tumour immunotherapies through DC modulation and engineering have shown great advancements in recent years [220]. DC cross presentation of tumour antigens initiates anti-tumour activity and clearance of tumours [221]. Studies showed that vaccination against melanoma both *in vitro* and *in vivo* using tumour antigenic peptides treated DCs have the ability to prime T cells generating an anti-tumour response [222, 223]. Another study by Springer et al. showed that DC based vaccinations supports anti-tumour chemotherapies in eradicating tumours [224]. This shows how engineered donor specific DCs are able to modulate the adaptive immune response translated in T cell differentiations towards specific phenotypes and result in anti-tumour activity. In this context in this chapter the aim is to understand the biological relevance of the monosaccharide treated DCs using DC-T cell co-culture experiments to study T cell differentiation to different functional subsets.

4.2 Methods

4.2.1 T cell isolation from PBMCs

As described in chapter 2 (section 2.6)

4.2.2 Sandwich ELISA

As described in chapter 2 (section 2.5.2)

4.2.3 RNA isolation and cDNA synthesis

RNA extraction and isolation were carried out using the RNeasy kit (QIAGEN) and cDNA synthesis was carried out using qPCR synthesis kit (PCRBIOSYSTEMS) according to manufacturer's instructions.

4.2.4 Real time-qPCR

mRNA extraction and isolation were carried out using the RNeasy kit (QIAGEN) and cDNA synthesis was carried out using qPCR synthesis kit (PCRBIOSYSTEMS) according to manufacturer's instructions. Real time PCR was carried out using SYBR Green qPCR Master Mix (PCRBIOSYSTEMS) and the qPCR cycler Stratagene MxPro 3005P qPCR System (Stratagene, La Jolla, CA). Primers used are GAPDH (forward) 5'-GAGTCAACGGATTTGGTCGT-3', GAPDH (reverse) 5'-GACAAGCTTCCCGTTCTCAG-3'; FOXP3 (forward) 5'-GTGGCCCGGATGTGAGAAG-3', FOXP3 (reverse) 5'-GGAGCCCTTGTCGGATGATG-3'; ROR γ t (forward) 5'-CCTGGGCTCCTCGCCTGACC-3', ROR γ t (reverse) 5'-TCTCTCTGCCCTCAGCCTTGCC-3'. The heat cycle was initiated at 95 for 2 min, followed by 40 cycles of 95 for 5sec, then 65 for 25sec and followed by 95 for 1min, 55 30sec 95 30sec. Sample values are relative to LPS only and are normalized to the house keeping gene GAPDH. All samples are run in triplicates.

4.3 Results

4.3.1 Investigating T lymphocyte populations purity and viability

Setting up DC-T cell co-cultures is generally challenging especially when it involves initial conditioning of DCs followed by setting up co-culture with T cells. In order to verify separation and isolation methods to obtain T cells from buffy coats, purity of isolated T cells was assessed using CD3 as a general marker for all T cells. Furthermore, CD4 and CD8 markers were used to confirm presence of both Th and T cytotoxic (Tc) population respectively and CD45RA was used to differentiate between the CD45RA⁺ naïve T cell and the CD45RA⁻ memory T cell populations. Data obtained (Fig.4.1) confirms the presence of CD3⁺ T cell population where both CD4⁺ Th and CD8⁺ Tc subtypes of T cells can be differentiated as well as natural killer cells which lack the expression for both markers. The expression of CD45RA shows the naïve T cell population while the lack of the expression shows the memory T cells population. This confirms the separation and isolation of T cells from buffy coats and the presence of the target populations to be used in further isolation of the naïve T cell population for DC-T cell co-culture experiments as well as our allergy model experiments.

T cell characterization after separation and magnetic bead isolation from buffy coat PBMCs

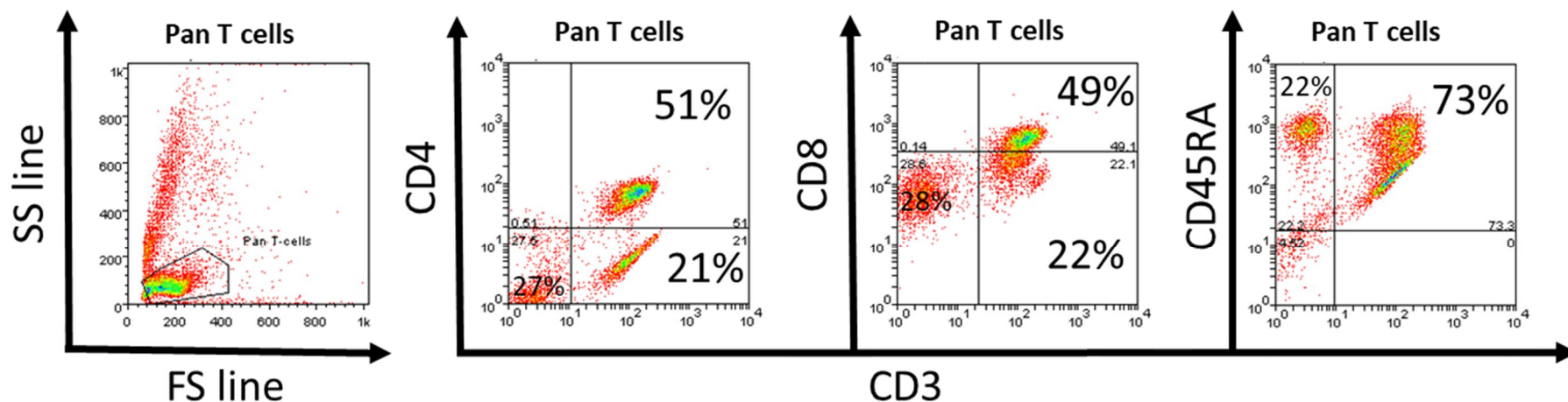


Figure 4.1: Flow cytometry characterization of isolated pan T cells from buffy coat PBMCs. The data suggests presence of both CD4+ and CD8+ populations of the CD3 T cells of over 70% with CD3- population corresponding to natural killer cells. Naïve T cells can also be differentiated from memory T cells with the expression of CD45RA+ in which both are present in the isolated pan T cell. Data shown are from a representative experiment of 2 donors

4.3.2 Investigating isolated naïve T lymphocyte purity

To obtain naïve T cells, further isolation steps are used on the pan T cell populations isolated from buffy coat PBMCs. This isolation focuses on depletion of CD45RO⁺ memory cells using microbeads and the remainder population of lymphocytes would be naïve T cells of both CD4⁺ and CD8⁺ subtypes. After the isolation process, T cells were analysed using the previously mentioned markers to assess presence of naïve CD3⁺ population of both CD4⁺ Th and CD8⁺ Tc and the absence of CD45RA⁺ memory T cells. Data in (Fig.4.2) confirm presence of CD3⁺ population of T cells expressing CD4⁺ or CD8⁺. Data also confirms the absence of the previously present population of CD45RA⁺ memory cells seen in (Fig.4.1) indicating the purity of the naïve cells obtained. Both CD4⁺ and CD8⁺ subtypes are present in the CD3 naïve T cell population. These cells can be used for the co-culture experiments to understand the downstream effects of monosaccharide primed DCs on T cell activation and immune system modulation.

T cell characterization after magnetic bead isolation from Pan T cells

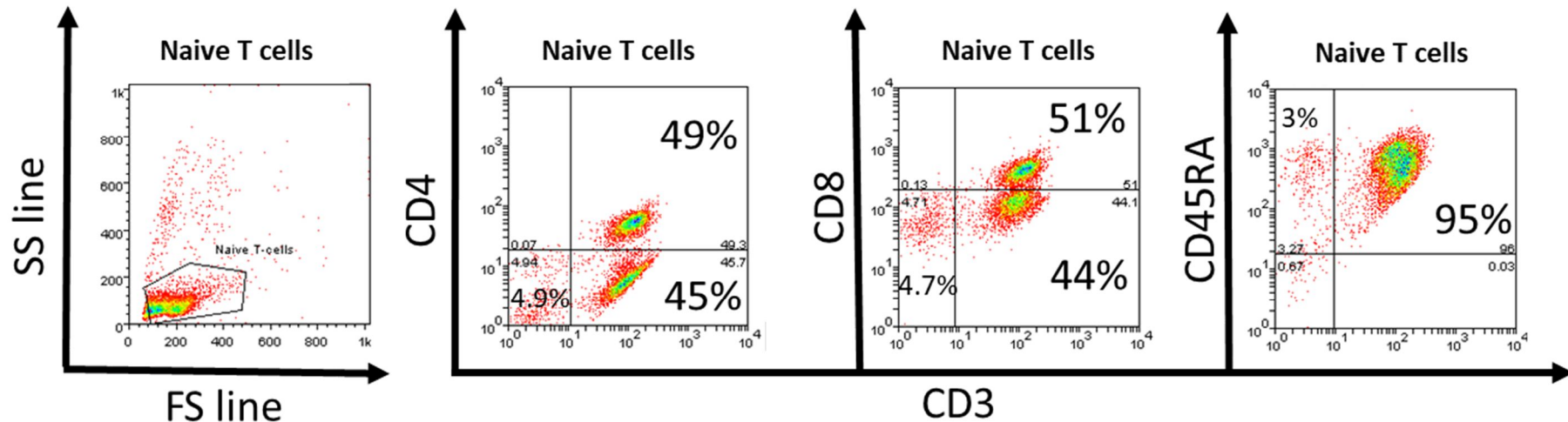


Figure 4.2: Flow cytometric analysis of naïve T cells isolation from pan T cell using CD45RO magnetic bead negative selection. A purity of 95% of naïve T cells expressing CD45RA was obtained showing successful depletion of memory T cells from the pan T cell population. Both subtypes CD4⁺ and CD8⁺ are of the CD3⁺ T cells are present. This confirms quality of the methods used for the isolation process of naïve T cells from pan T cells. Data shown are from a representative experiment of 2 donors

4.3.3 Viability and potency of liquid nitrogen preserved T-cells for autologous DC-T-cell in vitro co-cultures

To be able to co-culture primary cells, several hurdles must be overcome and most importantly is viability and stability of cells after freeze-thaw cycles. This is because we had to use autologous co-cultures which means that we need to use both DCs and T cell population obtained from the same donor. Preserving cells may hinder their ability to interact or artificially activate the cells leading to cell death and failed outcome of experiments. For that optimisation for preservation of Buffy coat isolated T cells for the duration of DC generation and condition treatment which is up to 7 days was addressed. The process used to preserve T cells until co-culture is illustrated in figure.4.3. Briefly, both CD14⁺ monocytes and T cells were isolated from the same donor where monocytes are incubated for differentiation towards DCs and T cells (naïve or pan) were preserved using liquid nitrogen (see materials and methods). After 7 days, thawed T cells were assessed for cell viability, activation, and co-culture behaviour with DCs was assessed using proliferation as an indicator of cell interaction. Viability was assessed after thawing from liquid nitrogen and after 6 days of co-culture with either monosaccharide prime DC or not and in the presence of LPS or not. T cell viability was done in both trypan blue automated counter and in flow cytometry Live/Dead assay (see materials and methods). Data in (Fig.4.4) show naïve and pan T cell viability using both trypan blue automated counter and live/dead flow cytometry assay. After 7 days of liquid nitrogen preservation pan T cells had a viability of 98.4% while naïve T cells had a viability of 93.2% of live cells. Comparing this to the after-isolation viability for both T cell groups show no significant reduction in number of live cells. Trypan blue stain using countess automated counter showed same viability for both groups of T cells confirming the results. This indicates that liquid nitrogen preservation methods used are not affecting T cell viability.

After 6 days co-culture, T cells showed a viability of >99% using Live/dead assay after co-culture with monosaccharides conditioned or not conditioned DCs in the absence or presence of LPS (Fig.4.5). This confirms the stability and the viability of T cells isolated for both naïve and pan populations. We also looked at the activation marker CD25 to understand if cells have been pre-activated before co-culture rendering them unable to interact with DCs which would provide false results. Data in (Fig.4.6) show population of both naïve and pan T cells isolated where CD25 an activation marker for T cells is not expressed confirming steady state of the cells obtained after thawing from liquid nitrogen.

We have then looked T cell proliferation after co-cultures with LPS activated DCs or not to confirm T cells potency in which preserved T cells should still able to interact with DCs and proliferate. Naïve T cells lack the memory subtype and are slower to proliferate, for that they were re-stimulated with anti-CD3 and anti-CD28 while pan T cells were not re-stimulated.

Proliferation graph (Fig.4.7) show that isolated T cells for both naïve and pan were able to proliferate upon co-culture with mature DCs confirming their quality and potency after liquid nitrogen thawing.

This proves that methods used for the isolation of both pan and naïve T cells produced good quality cells and the preservation methods for these cells can be utilised to further move towards autologous DC-T cell co-culture experiments.

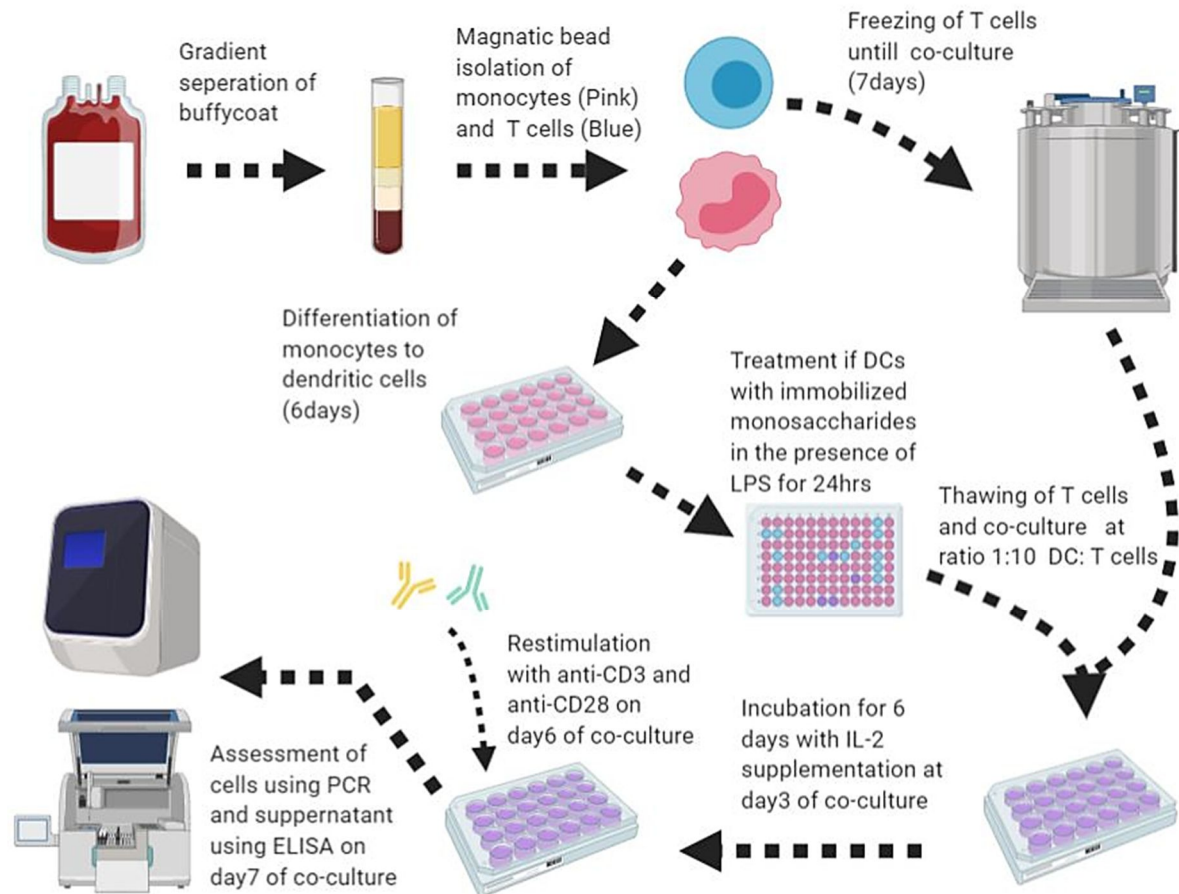


Figure 4.3: Illustrating process of preservation of T cells and autologous DC-T cell co-culture. In this process both monocytes and T cells are isolated from the same donor using magnetic beads after gradient separation from buffy coats. T cells are stored in liquid nitrogen for 7 days while monocytes are differentiated to DCs (6 days) and treated with immobilized monosaccharides (24 hrs). T cells are thawed and co-cultured with DCs at 1:10 ratio of DC:T cells. At day3 of co-culture the cells are supplemented with IL-2 and at day6 they are restimulated with anti-CD3 and anti-CD28 for 24 hrs. Assessments are carried out at day 7 for PCR and ELISA.

Comparing T cell viability after isolation process and after liquid nitrogen preservation using flowcytometric Live/Dead assay and Trypan blue automated counter

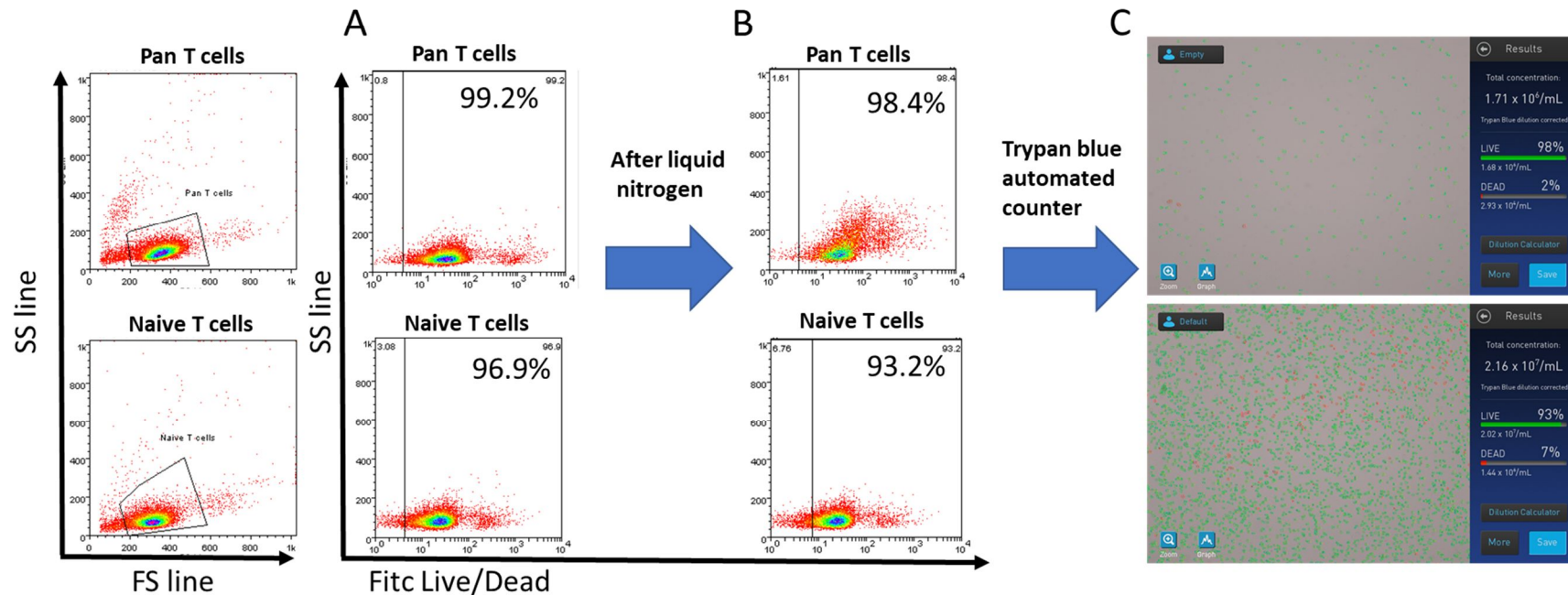


Figure 4.4: Showing viability for both pan and naïve T cells after the isolation process and liquid nitrogen using dead/live flow cytometry staining and Trypan blue automated counter. A) Data suggested viability of 99% for pan T cells after isolation from healthy donors' buffy coat PBMCs. Subsequent isolation of Naïve T cells from the pan T cells populations has a viability of 96.9% of live cells. B) Comparing after isolation viability with after liquid nitrogen thawing of T cells from liquid nitrogen shows no significant decrease in viability of live cells. C) The data obtained is also consistent with trypan blue automated counter showing similar T cell viability for both naïve and pan T cells. Data shown are from a representative experiment of >2 donors for live/dead flow cytometry assay and >8 donors for Trypan blue automated counter.

Determining T cell viability after autologous cocultures with DCs using flowcytometric Live/Dead assay

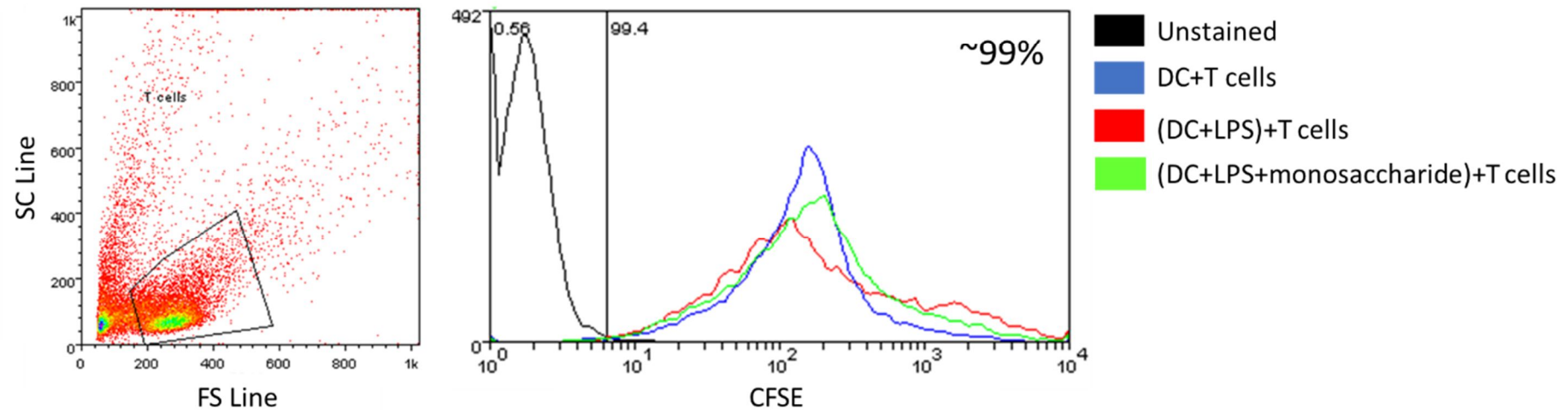


Figure 4.5: Flow cytometry live/dead assay naïve T cell viability after 6 days of autologous DC co-culture. Data indicates a viability of >99% of live cells after 6 days of co-culture. This suggests that viability of naïve T cells was not affected by the 6 days of co-culture processing after preserved in liquid nitrogen confirming the quality of cells. Data shown are from a representative experiment of one donor for live/dead flow cytometry assay.

Determination of T cell activation after revival from liquid nitrogen

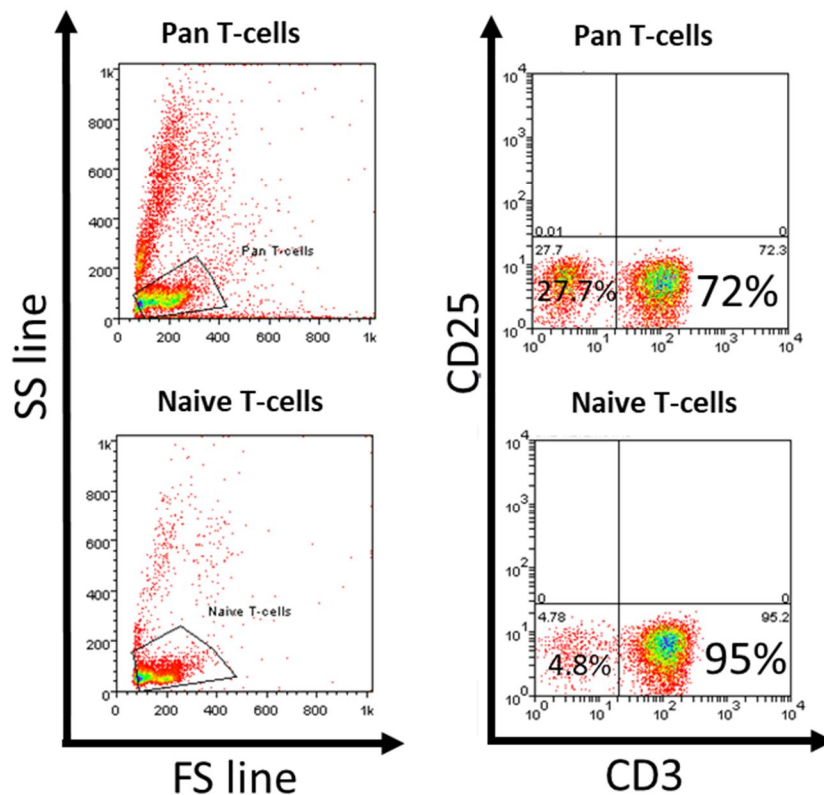


Figure 4.6 Flowcytometry data illustrating CD25 expression in the CD3+ T cell population to determine if preservation activates cells. Data shown for both pan T cells and naïve T cells for both CD4+ and CD8+ populations. CD25 activation marker for T cells was not expressed after liquid nitrogen preservation of cells for 6 days. This shows that liquid nitrogen preservation method does not artificially activate both pan and naïve T cells and can be used for the preservation of T cells for subsequent autologous DC co-cultures. Data shown are from a representative experiment of 2 donors

Determining T cell ability to interact with DCs in autologous cocultures after preservation using proliferation assay

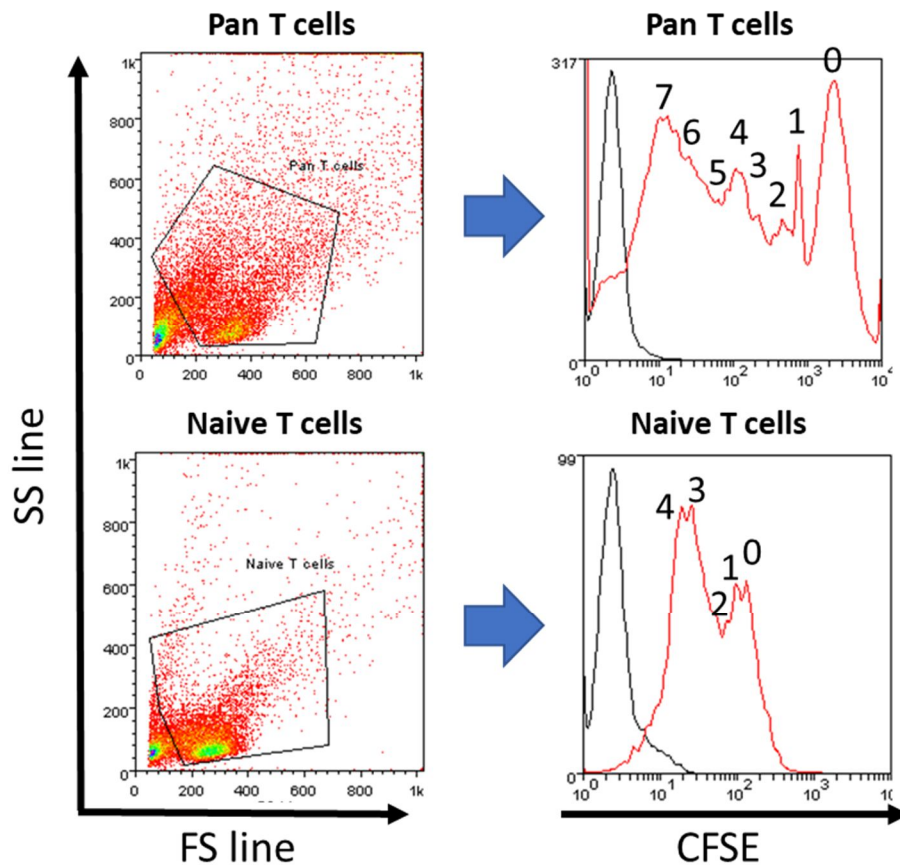


Figure 4.7: Proliferation assay was carried out on both pan and naïve T cells using CFSE live cells staining. Pan T cells were co-culture for the duration of 6 days and then analysed while naïve T cells were re-stimulated on day 6 with anti-CD3 and anti-CD28 for 24hrs and analysed on day 7. Data show multiple peaks each representing a generation of proliferated T cells starting from the origin which is marked as 0. Pan T cells proliferated 6 generation in 6 days while naïve T cells proliferated 3 generations in 7 days. Data shown are from a representative experiment of 2 donors for CFSE flow cytometry assay.

4.3.4 DCs conditioned by 100%Gal1 & 50%Gal1 50%Gal2 bias T cell differentiation towards IL-10 and IL-17 producing cells respectively

Monosaccharide combinations Gal1, Man1-Man2, Man1-Gal2, Man2-Gal2 and Gal1-Gal2 induced high levels of IL-10 while suppressing production of IL-12 and IDO activity in LPS stimulated DCs. These conditions were selected for investigating downstream effects on T cell differentiation in DC-T cell co-culture studies. Co-cultures were carried out using autologous naïve T-cells and DCs where DCs were primed with the selected carbohydrates in the presence of LPS for 24hrs and then co-cultured with T cells for 7 days. On day 6 T cells were re-stimulated with anti-CD3 and anti-CD28 for 24hrs where on day 7 cytokine profile is assessed. Data show a significant increase in IL-10 production by naïve T-cells in Gal1, Gal1-Gal2 and Man2-Gal2 conditions (Fig.4.8). An interesting finding was T cells co-cultured with Gal1 and Gal1-Gal2 primed DCs show a significant increase in IL-17 production compared to LPS alone whereas a decrease in IL-17 can be noted in T cell co-cultured with DCs primed with Man1-Gal2. Significant reduction of IL-4 in conditions Gal1-Gal2 and Man2-Gal2 can be seen while IFN- γ levels are comparable to that of unconditioned.

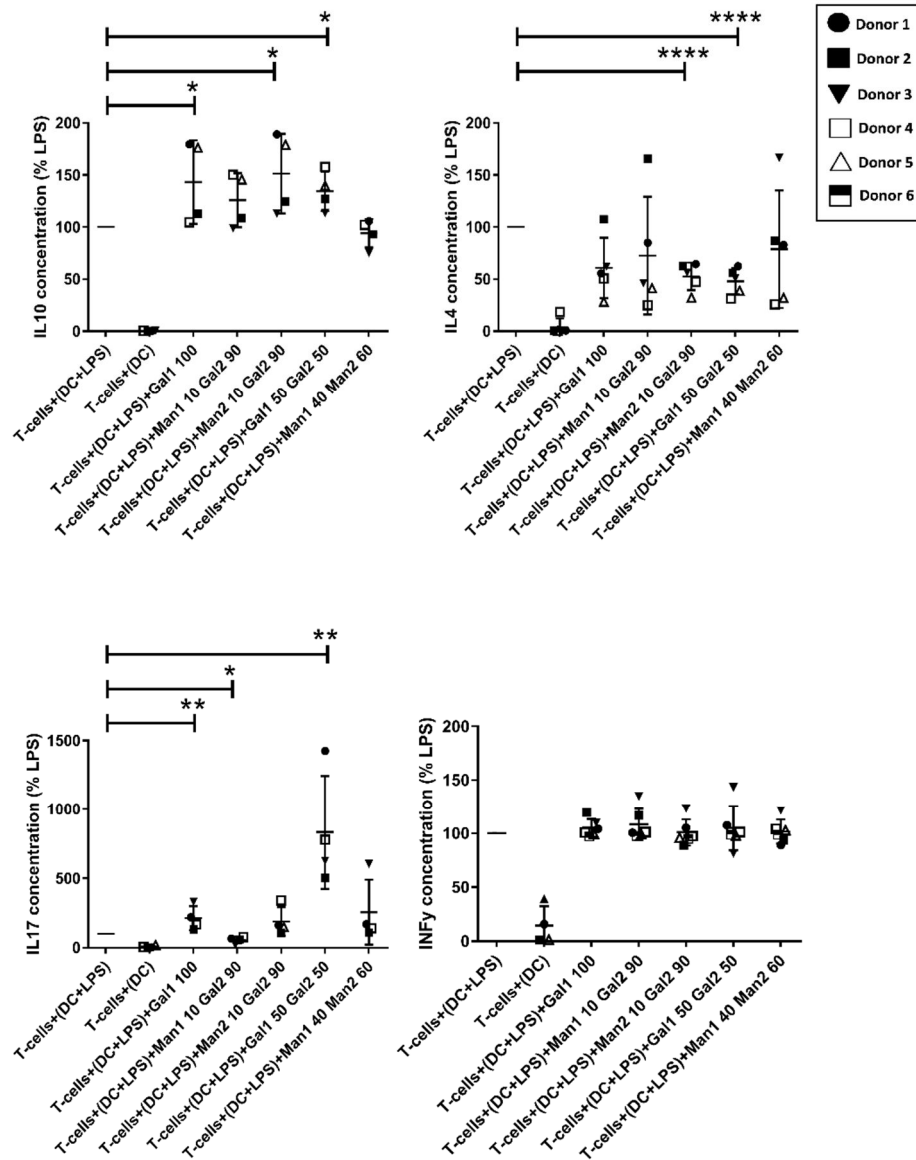


Figure 4.8: Naïve T cell cytokine profile using ELISA obtained after co-culture with monosaccharide conditioned DCs. The cytokine profile was assessed for (IL-10, INFγ, IL-4 and IL-17) to look at the downstream t cell phenotype has gone through. An increase in IL-10 production can be noted in all conditions where significant in 100% Gal1, 10%Man2+90%Gal2 and 50%Gal1+50%Gal2. Interestingly the same conditions show a great increase in IL-17 productions most significant in 50%Gal1+50%Gal2 indicating a possible presence of Th17 and T-reg in the population or a double expression of the later in the same cells. A significant decrease in IL-17 can be noted for 10%Man1+90%Gal2 showing a shift of the immune system away from Th17 and into Th1. Data is shown as mean $\pm$ SD of ≥ 4 independent donors where *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4.3.5 DC conditioned with Gal1 or Gal1-Gal2 promote T cells with mixed Th17 and T-reg phenotype while Man1-Gal2 primed DCs suppress Th17 phenotype in DC-T cell co-cultures

From this data we hypothesised the presence of T cell populations expressing both anti-inflammatory and pro-inflammatory phenotypes which have been recently described in the context of chronic inflammations and cancers. To further confirm the phenotype of these cells we investigated expressing of FOXP3 and ROR γ t, major T-reg and Th17 transcription factors respectively, using real time polymerase chain reaction (RT-PCR). We were able to confirm the expression of both FOXP3 and ROR γ t indicating the generation of cells with a mixed T-reg/Th17 phenotype on immobilised Gal1 and Gal1-Gal2 (Fig.4.9). Interestingly, Man1-Gal2 showed a decrease in IL-17 production and ROR γ t gene expression showing that the incorporation of mannose changed cell differentiation away from Th17 towards Th1 phenotype. This provides evidence for the potential of the carbohydrates in monosaccharide forms to modulate the immune system and open new possibilities in use of such approaches in treatment and management of inflammatory conditions.

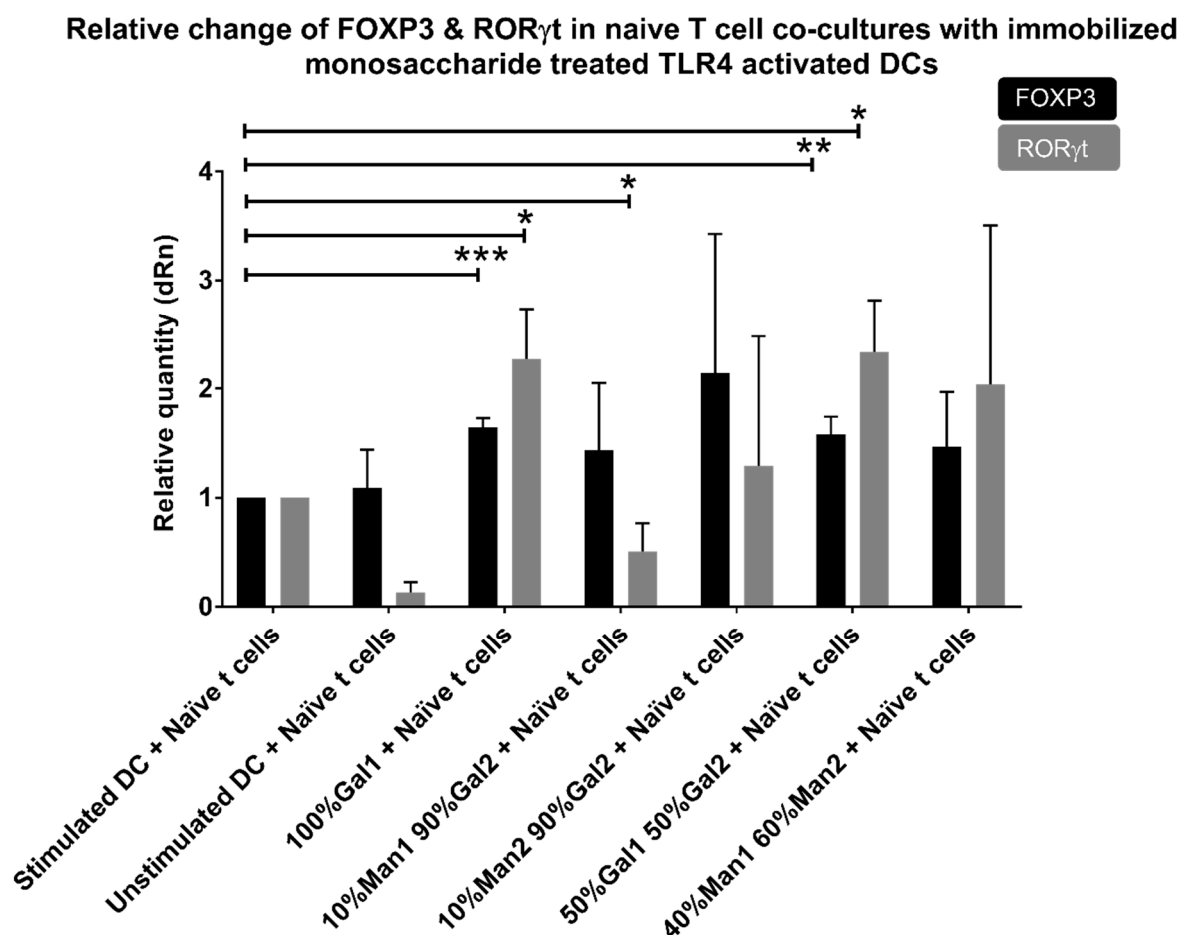


Figure 4.9: RT-PCR data obtained from DC-T cell co-cultures where DCs are primed by the immobilized monosaccharides and then co-cultured with autologous T cells. The gene expression assessed for (FOXP3 and ROR γ t) to further confirm t cell phenotype. Significant increase in FOXP3 expression can be noted in all conditions where significant in 100%Gal1 and 50%Gal1+50%Gal2. Interestingly a great increase in ROR γ t production can be noted in 50%Gal1+50%Gal2 and 100%Gal1 indicating a possible presence of Th17 and T-regs in the population or a double expression of both genotypes in the same population. A decrease is in 10%Man1+90%Gal2 indicating that the incorporation of mannose decreased Th17 presence. Data is shown as mean $\pm$ SD of 3 independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.6 DCs conditioned by 10%Man1+90%Gal2 bias T cell differentiation towards Th1 instead Th2 in house dust mite allergy model

Our previous observations on DCs conditioned with Man1-Gal2 showed a significant decrease in Th17 population compared to other conditions and LPS only control. This is seen through the significant decrease in IL-17 secretion by co-cultured naïve T cells. This finding has been confirmed by RT PCR where a reduction in the gene ROR γ t which is expressed during the differentiation of T cells towards Th17. Lowering Th17 is beneficial in reducing chronic inflammation, chronic allergic reactions as well as possible implications that link Th17 with progression of tumours. In this context, we have looked at the effect of DC conditioned with 10%Man1+90%Gal2 on T cell differentiation in the presence of house dust mite (HDM) allergen extracts and LPS. The presence HDM extract is used in allergy models to bias immune response towards Th2 profile. This experiment will reflect the potential of the immobilized monosaccharide conditioned DCs to change the outcome of the chronic allergy model by modulating T cells.

Cytokine data obtained from the allergy model show a decrease in IL-17 for the condition Man1-Gal2 with no changes in IFN γ and IL-10 (Fig.4.10). This is compared to our previously confirmed condition with increased Th17 and T-reg population Gal1-Gal2 where the outcome did not change than the previous observations. This indicates that the decrease in IL-17 is genuine reducing presence of Th17 T cells and proving modulatory property of the condition Man1-Gal2.

Determining pan T cell cytokine profile in HDM allergy module after treatment with DC primed Man1-Gal2 or Gal1-Gal2

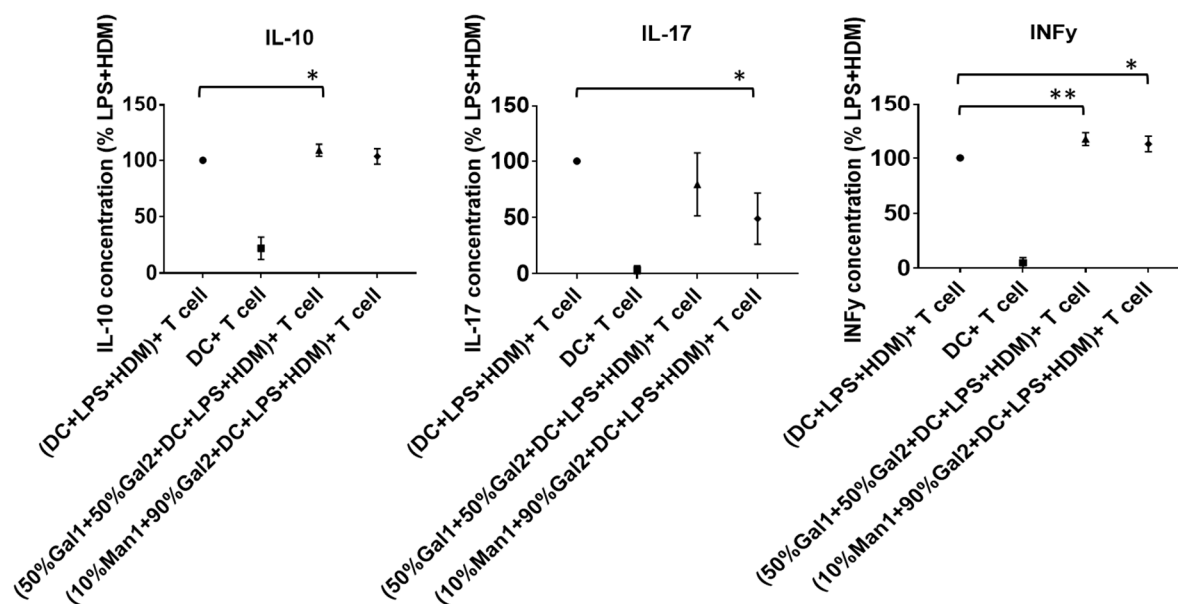


Figure 4.10: Determination of pan T cell cytokine profile after co-culture with DC primed with 10%Man1+90%Gal2 or 50%Gal1+50%Gal2 compared to LPS+HDM as an allergy model. Data show a significant decrease in IL-17 and an increase in INF γ for 10%Man1+90%Gal2 which supports our finding in the naïve co-culture model. This is also the case for 50%Gal1+50%Gal2 where there is no decrease in IL-17 and a significant increase in both IL-10 and INF γ . This suggests that 10%Man1+90%Gal2 decreased the Th17 content and shifting T cell phenotype towards Th1 in the allergy model and in result would decrease the chronic reaction by both LPS+HDM. Data is shown as mean $\pm$ SD of 3 independent donors where * $p < 0.05$, ** $p < 0.01$.

4.4 Discussion

The biological importance of immobilized monosaccharide are yet to be explored, despite our understanding of the modulatory properties of complex carbohydrates on the immune system [225]. The functionality of complex carbohydrates in modulating the immune system has been shown by many studies. These studies provide wider understanding in the field, where modulated DCs are able to migrate to lymph nodes and interact with T cells and skew the immune response towards desired phenotype. A study by Jin H. Kim. et. al. showed that reprogrammed DCs through transfection are able to migrate and cross present antigens to T cells *in vivo* [226]. Another study found that modulated DCs with high inflammatory IL-12 content were more potent in expressing melanoma antigen and migrating to lymph nodes [227]. These studies show the importance of understanding the biological relevance of modulated DCs and how it is linked to guiding the adaptive immune response.

Downstream effects provide the whole picture where we can understand the modulatory properties of monosaccharides primed DC on shaping the adaptive immune response.

We started by looking at the cytokine profile of naïve T cells co-cultured with our selected immobilized monosaccharide primed DCs. Data obtained showed an increase in IL-10 most significant for conditions Gal1, Gal1-Gal2 and Man2-Gal2. Reduction in IL-4 is significant in Gal1-Gal2 and Man2-Gal2 while there are no changes in INF γ . This indicates the presence of a regulatory phenotype with a greater shift towards Th1 phenotype compared to other conditions and control. This is in line with our previous findings in chapter 3 where conditioned DCs expressed a regulatory phenotype through increased IL-10 production and reduced IDO activity. Research have shown that Leishmaniasis reduce expression of CD40 while increasing IL-10 and INF γ production shifting T cells towards inactive Th1 phenotype ultimately causing the persistence of the parasite [228]. These parasites are known to interact with MR on DC

through mannosylated glycoprotein surface [229]. An intriguing finding was that high levels of IL-10 is accompanied by high levels of IL-17 which is unique for Gal1 and Gal1-Gal2. This suggests the presence of both Th17 and T-reg in the population of T cells which may indicate the presence of plastic T cells that express both genotypes and are able to play both roles of anti-inflammatory and pro-inflammatory simultaneously or a greatly suppressive phenotype related to suppressing Th17. This is believed to be due to presence of IL-23 in the micro environment produced by DCs which reprograms T-reg towards co-expressing ROR γ t [230]. Research show that these cells originating from a T-reg precursor maintain their suppressive effects and control Th17 induced inflammation [218]. Research shows that hybrid T cell population expressing both FOXP3⁺ and ROR γ t⁺ are a stable phenotype and have greater suppressive potential than FOXP3⁺ only T-reg [231]. The increased suppressive effects and their contribution to gut immune stability have also been proven by *in vivo* studies [232]. This hybrid population have also been hypothesised to be related to the pathogenesis of pancreatic cancers through their suppressive ability [233-235]. That being said the addition of mannose to galactose combination showed the opposite of that effect where a significant decrease in IL-17 production while maintaining a relatively high IL-10 levels. There are currently no evidence supporting our finding of Man1-Gal2 reduction in IL-17 content in T cells and so we have decided to move to additional experiments to further confirm our findings using gene expression and an allergy model which will be discussed further in this chapter.

Looking at the conditions Man1-Man2 and Man2-Gal2 cytokine profiles suggests a pro-inflammatory phenotype rather than regulatory phenotype. Man1-Man2 show no changes in IL-4 while Man2-Gal2 show a significant reduction in IL-4 production by the naïve T cells. This suggests that Man1-Man2 is a more pro-inflammatory towards both Th1 and Th2 while Man2-Gal2 shifts immune response towards a regulatory and Th1 protective phenotype. Given the combinations of both monosaccharides this is consistent with literature where increase in mannosylation in organisms would induce more pro-inflammatory phenotypes such as

Mycobacterium mannose capped glycoproteins [236] while galactose incorporation into the glycoproteins would promote a suppressive phenotype such as that found in tumours interaction with MGL on DCs [237].

Next, we looked into confirming our findings through looking at gene expression in each condition to link both ends (gene expression and cytokine secretion) of the generated immune response. PCR data showed marked increase in the regulatory gene FOXP3 in conditions Gal1 and Gal1-Gal2 which have previously shown marked increase in IL-10. This confirms the increase of the presence of a regulatory phenotype in these conditions compared to control. Interestingly, both of these conditions have also showed increased expressions of ROR γ t compared to other conditions. Similar population of T cells have been found in B7.1wa mice cells treated with CTL enhancements to create engineered DCs [238]. These consistent findings with the fact that both conditions are combinations or pure galactose are in line with studies showing galactose containing organisms such as candida have a similar response from the immune response in generation of IL-17 secreting T-regs [239]. It is remarkable that glycoproteins on these fungi utilizing galactose to modulate immune cells through lectins which we have shown similar effects through our immobilized galactose combinations.

Using our allergy model through introducing HDM and LPS to promote an inflammatory DC phenotype that would skew T cells towards Th2 [240]. This is done while conditioning on Man1-Gal2 that have shown a decrease in IL-17 and Th17 expression to understand if DCs primed by this condition prove effective in lowering chronic Th17 induced allergy. This is done in comparison to Gal1-Gal2 which had an increase in IL-17 and Th17 and unconditioned controls. Data shown in figure 9 indicate that Gal1-Gal2 behaves similarly in the allergy model increasing IL-10 while having unchanged IL-17 production. This proves consistency of our results for the condition Gal1-Gal2. The outcome for Man1-Gal2 show remarkable reduction in IL-17 suggesting modulatory properties of this combination in possible lowering of Th17

and chronic inflammation in allergies. Both conditions have increased INF γ indicating favourable protective Th1 shift compared to control where outcomes are more towards Th2 allergic response. This together shows that Gal1-Gal2 and Man1-Gal2 have induced a more protective Th1 phenotype in our model with the later condition having reduced Th17 content and thus lowering chronic inflammation. The increase in IL-10 in Gal1-Gal2 may also indicate a more regulatory phenotype that produce IL-17 controlling chronic inflammation. These findings while being supported by other studies still needs more experimentation and research to reach more understanding of the immobilized monosaccharides immunomodulatory ability and potential that can be harnessed in regenerative medicine and controlling inflammation through coatings and implants.

4.5 Conclusions

This chapter discussed the biological relevance of our monosaccharide primed DCs in modulating the adaptive immune response. Engineered DCs are means of modulating the adaptive immune response to attain desirable pro-inflammatory and anti-inflammatory outcomes. DCs treated with our immobilized monosaccharide conditions Man1-Gal2 and Gal1-Gal2 were able to modulate the TLR4-CTL crosstalk attaining a different DC phenotype then LPS alone. This was seen through the co-culture experiments and allergy models in which these DCs were able to interact with naïve or pan T cells and induce a lower Th17 or increase Th17 populations for Gal1-Gal2 and Man1-Gal2 respectively. These immunomodulation properties of monosaccharides primed DCs on T cells is novel as immobilized monosaccharides have not been studied as immune modulators for immune cells of the innate and adaptive responses. This provides enough evidence of the immunomodulatory properties of the monosaccharides in immobilized form and brings insights into the development of new therapies to lower inflammation using Gal1 and Gal1-Gal2, lowering chronic inflammation for regenerative medicine using Man1-Gal2 and

vaccinations through increasing inflammation for anti-tumour therapies using Man1-Man2 and Man1-Gal2.

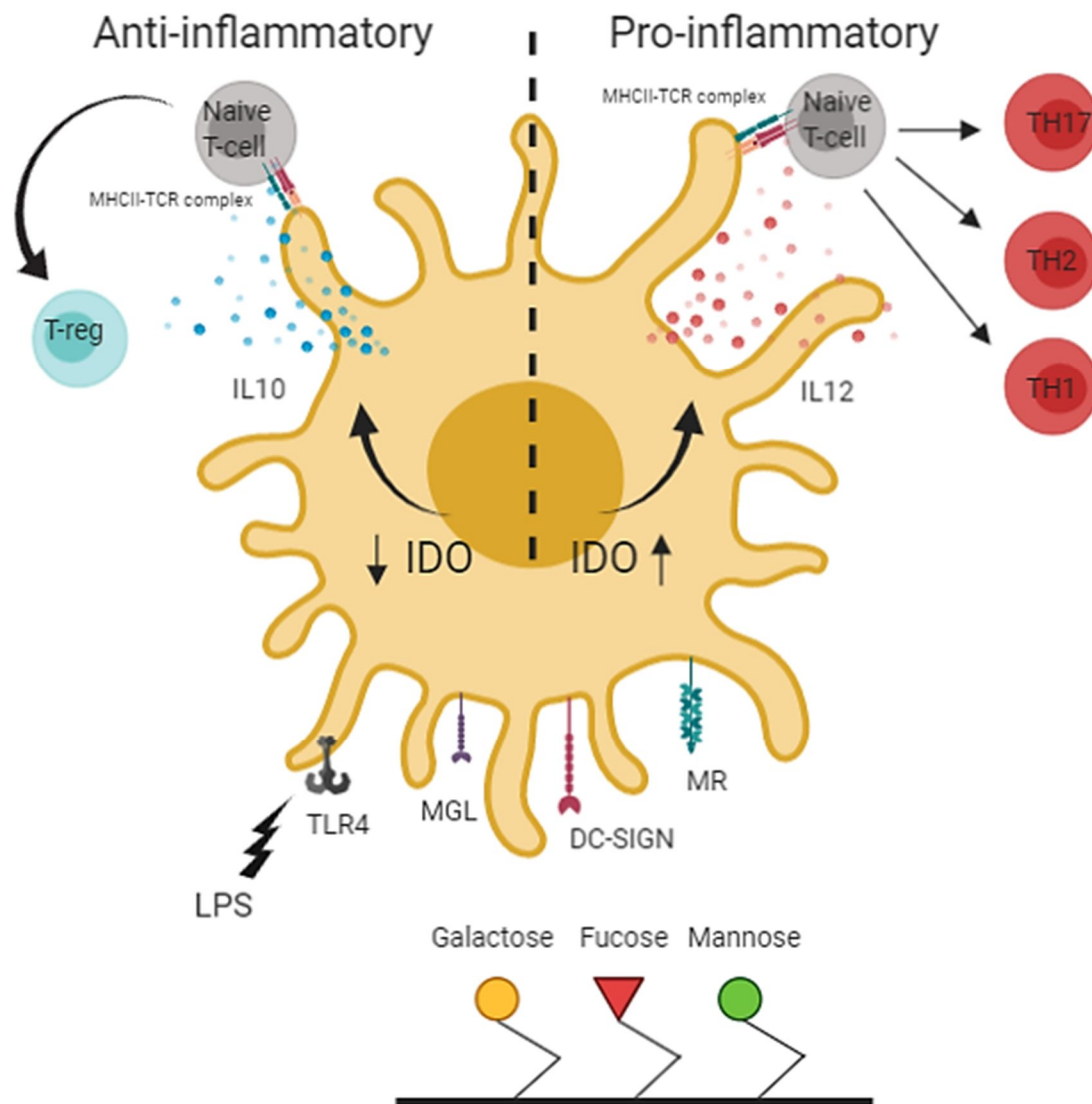


Figure 4.11: Diagram showing interactions between DC surface lectins and Immobilized monosaccharides in the presence of a TLR4 ligand (LPS). TLR4 induced response is modulated affecting IDO enzyme activity and in turn resulting in skewing immune response in respect to the cells cytokine profile and co-stimulatory marker expression. This determines the downstream effect on T-cell polarization and differentiation leading to distinct phenotypes and ultimately the direction the immune system's response.

Chapter 5:

Fucose coated nanoparticles: enhanced uptake and rapid lysosomal delivery

5.1 Introduction

Nano Particles (NPs) have been extensively studied as means for drug delivery for vaccination and immune modulation [241]. NPs are defined as ultrafine objects “particles” of diameters less than 100nm [242]. Nanotechnology have been gaining attention in the field for their properties attributed to the small size in many fields including cancer therapies and drug delivery [243]. This minute size allows these particles to infiltrate different kind of tissues distributing itself. This is particularly important in terms of fighting solid tumours for example in which NPs were proposed in medicine as anti-cancer drug delivery intermediates [244]. Current technology advancements have facilitated shaping and synthesising NPs from different materials. Studies have shown that NPs of different shapes, chemistries and sizes have different outcomes on the cellular level specifically on immune cells [245]. Gold is one of the materials that is used for NP fabrication because of its inert chemical properties as well as physical stability [246]. Gold nanoparticles (AuNPs) have been shown to have no toxic effects *in vivo* which in turn promoted more research to utilize them for drug delivery and other medical application [247].

As discussed in previous chapters DCs act as the modulators of the adaptive immune response. The migratory capacity of DCs makes them the ideal candidates for drug delivery and immune modulation [248]. Using NPs for DC drug delivery have been successfully achieved by many studies. Studies concentration on AuNPs drug delivery showed that AuNPs with polyelectrolyte multilayer coatings increased DCs ability to present antigens to CD8 T cells [249]. Another study showed that AuNPs coated with small interfering RNA can be successfully delivered to the nucleus through endosomal escape mechanisms utilising PH and charge modifiers [250].

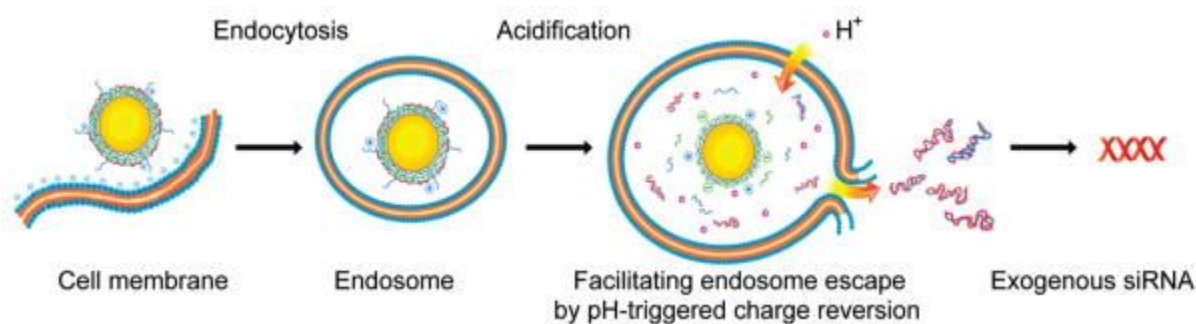


Figure 5.1: AuNP escape from endosomal compartments using PH modifiers for the delivery of siRNA to the nucleus. Adapted from [250].

There are many mechanisms for drug delivery using AuNPs in order to achieve proper intracellular concentrations of the drugs or mediators [251]. Recently, glycans gained attention in the field of drug delivery where they have been utilized to delivery payloads to APC specifically DCs [252].

Glycans are abundantly expressed on cellular surfaces however they are specific to the type of tissue they originate from and the species. Tumours are known to express aberrant glycosylation patterns which in turn would be identified by the immune system and terminated [253]. However, cancers ability to suppress the immune system stopping APCs from performing their function thus promoting the tumours survival [253]. Antigen delivery using nanoparticle payloads have been studied to overcome this limitation creating new means for immunization against cancers [254]. CTLs enable APCs to differentiate between self and foreign antigens through their monosaccharides and glycans. These CTLs are the one of the main mechanisms which APCs differentiate foreign or aberrant antigens from self-antigens. Glycan coated nanoparticles have been proved to bind to CTLs and take an intercellular route towards antigen presentation and adaptive immune activation. Studies have shown that DCs challenged with glycan functionalized nanoparticles coated with tumour antigen payloads are able to cross present the tumour antigens and activate CD8 T cells

initiating a cytotoxic effect against tumours [255]. These methods have also been studied in creating personalized medicines creating individualized cancer treatments [256].

Dextran is a member of the polysaccharide family have been studied as potential coating for AuNPs for anti-tumour drug delivery directly to cancer cells [257].

Another study shows that AuNPs coated with Streptococcus glycoconjugates is a promising mean for vaccination [258]. Sukyung Ahn et al. showed that AuNPs coated with tumour antigens were able to promote activation of CD8 T cells through DCs priming and activation [259].

To achieve optimal drug delivery and desirable concentration, uptake of particles has been studied [260]. By understating particles rout of uptake whether it is phagocytosed passively “micropinocytosis” or actively such as clathrin or coveoli mediated, we can determine the fate of the payloads [261]. It has been shown that AuNPs can be distributed through tissues and into cells through passive uptake mostly because of their attributed size [262]. This is changed when the particles are coated with substances to engage cell receptors such as antibodies, antigens or other biological coatings [263]. The method of uptake is important for vaccination for the delivery of the payloads to targeted cells and through the antigen presentation pathway [264]. This is achieved through the active pathway in which cells receptors recognizes the coatings and uptake the particles.

Vaccination is reliant on antigen delivery to target APCs in which the antigen is internalized processed and presented to the adaptive immune cells [265]. The phagosome containing the antigen fuses with a lysosome and is degraded and represented on MHCs on the cells surface. This efficient antigen presentation process requires sufficient uptake of the antigen as well as rapid localization in phagosome-lysosomal compartment to invoke greater presentation of the antigen.

In this chapter we look at monosaccharide coated gold nano particles (sAuNP) to identify optimal monosaccharide combinations that facilitate delivery and uptake of NPs by dendritic

cells and the impact on target cells namely DCs. Moreover, we look at trafficking of payloads towards lysosomal and endosomal compartments using monosaccharides as means of antigen delivery for vaccinations and various RNA silencing therapies. Three different monosaccharides (mannose, galactose and fucose) were used in combinations coated on 16nm colloidal spherical gold nanoparticles in the screening process where cellular uptake and phenotype were studied as well as localization of the sAuNPs into the cell's micro compartments. Understanding such mechanisms facilitates the use of simple carbohydrates in drug delivery to DCs for the development vaccines including anti-tumour vaccines

5.2 Methods

5.2.1 Synthesis and Characterization of Polymer-Coated Gold Nanoparticles

Gold chloride trihydrate (HAuCl_4 ; > 49% Au, ACS grade) and thiol-terminated PEG ($M_n = 3000$ g/mol) were purchased from Sigma-Aldrich. Trisodium citrate (99.8%) was purchased from Acros Organics. Ultra-high-quality water with a resistance of $18.2 \text{ M}\Omega \times \text{cm}^2$ (at 25°C) was obtained from a Millipore Milli-Q gradient machine fitted with a $0.22 \mu\text{m}$ filter. All other chemicals were used as received unless otherwise indicated [137].

Synthesis, characterization and coating of AuNPs was performed in Warwick University by our collaborators Dr. Matthew I, Gibson and Dr. Sarah-Jane Richards.

5.2.2 Synthesis of Citrate-Stabilized Gold Nanoparticles.

The synthesis of 16 nm diameter of gold nanoparticles is described. Briefly, 600 mL heated solution of a 0.85 mmol/L aqueous solution of HAuCl_4 to reflux in a scratch-free round bottomed flask. Then a ratio of 1:3 of Au/sodium citrate is made by adding 10.5 mL of a 0.5 mol/L of sodium citrate solution was added in a single portion to the HAuCl_4 . The temperature was maintained at reflux for 30 min, during which time a deep-red coloration formed. The reaction mixture was then allowed to cool to room temperature over a period of 3 h. Assuming complete reduction of the HAuCl_4 into the particles, the total gold concentration in the final solution was 0.17 mg/mL [137].

5.2.3 Physical and Analytical Methods.

UV–visible absorbance spectra were obtained on a Varian Cary 100 Bio spectrophotometer operating at 25°C using 10 mm path length cuvettes with a total volume of 2.5 mL. The method of Fernig and coworkers was used to estimate the diameter of citrate-stabilized gold nanoparticle using UV–visible spectroscopy [138].

Dynamic light scattering measurements were carried out on a Brookhaven Instruments system consisting of a BI-200SM goniometer and a BI-9000AT autocorrelator. A 100mW Ar⁺ ion laser (Lexel Lasers) operating at 488 nm was used. All measurements were performed at a temperature of 25.0 (±0.2) °C and at a scattering angle of 90°. Borosilicate cuvettes were used with a minimum of 3 mL of the nanoparticle solution. The nanoparticles were used at a concentration of ~0.05 mg/mL (total gold mass) in Milli-Q water and passed through a 0.4 µm filter before measurement [137].

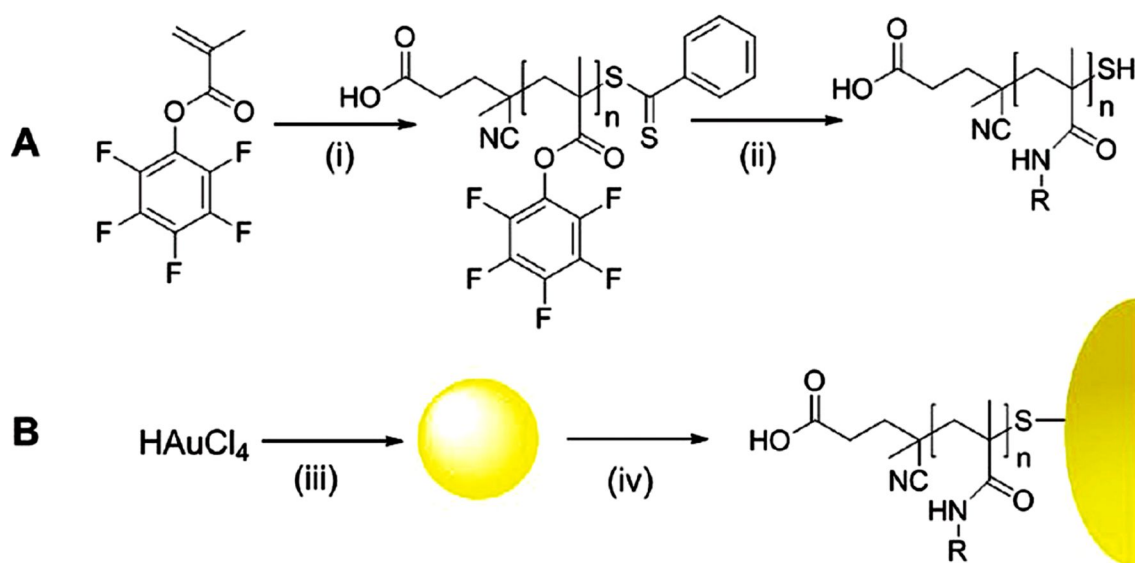


Figure 5.2: Illustrates functionalization of gold nanoparticles where A) is the substitution of pentafluorene with thiol B) Anchoring of the thiol to the gold nanoparticle. This figure was adopted from [139].

5.2.4 Gold nanoparticles monosaccharide coating

Approximately 10 mg of the desired thiol-terminated amino-monosaccharide was dissolved in 0.5 mL of high-purity water with the pH adjusted to ~10 using 0.1 M NaCl solution. To this tube, 120 mL of the citrate-stabilized gold nanoparticle solution (0.85 mmol/L total gold concentration) was added and then agitated overnight in the absence of light. To remove excess unbound monosaccharides, the particles were centrifuged for 3 h at 8000 rpm.

Following careful decantation of the supernatant, the particles were then redispersed in 20 mL of high-quality water, and the centrifugation–resuspension process was repeated for a total of three cycles. After the final cycle, the particles were dispersed in 10 mL of high-quality water for future use. Assuming complete incorporation of the citrate-coated gold particles into the final polymer-coated particles, the total concentration of gold in the final solution was 10.2 mmol/L (2 mg/mL)

5.2.5 Monocyte and naïve T cell isolation

As previously mentioned in chapter 2, monocytes were isolated from buffy coats of healthy donors in accordance with the ethics and approval of National blood bank, Sheffield, U.K.

5.2.6 Preparation of AuNPs and cell culture

Gold nanoparticles were suspended by centrifugation with 8000rpm for 30 minutes. Excess water is aspirated, and the pellet is resuspended in RPMI 1640 supplemented with 10%FBS, 100µg/mL streptomycin, 100U/mL penicillin and 2mM L-glutamine (all from Sigma-Aldrich). 100ul of each of the resuspended gold particles are added to 400ul of supplemented medium containing 300k dendritic cells. Incubation is carried out in 37° in a 5% CO₂ humidified incubator overnight where they are analysed the following day.

5.2.7 DC-T cell coculture

Autologous co-cultures were carried out with monosaccharides primed DC as previously described in chapter 2. Briefly, Pan T cells were isolated from buffy coat PBMCs using an immunomagnetic kit followed by naïve T cells separation using CD45RO immunomagnetic beads (both from Miltenyi Biotec), and co-cultured with DC at a 1:10 DC to T cell ratio in RPMI1640 supplemented with 5% human AB serum 100µg/mL streptomycin, 100U/mL penicillin and 2mM L-glutamine (all from Sigma-Aldrich) On day 3 cells are supplemented with IL-2 2ng/ml and on day 6 cells are stimulated with anti-cd3 and anti-cd28 overnight where they are ready for analysis on day 7 [136].

5.2.8 Analytical Flowcytometry

Cells were harvested and washed with PBA (PBS with 0.5% bovine serum albumin and 0.1% sodium azide). Incubation with labelled antibodies (MHC2 APC, MHC1 PE, CD86 FIT-C) (Miltenyi biotech) was then carried out in the dark at 4°C for 20 minutes. Cells were then washed in PBA and fixed with 4% paraformaldehyde until analysis. All samples are analysed using FC500 Flow cytometer (Beckman Coulter) and data analysis was carried out using Weasel software.

5.2.9 Membrane cholesterol depletion

Dendritic cells were treated with 10mM of Methyl- β -cyclodextrin suspended in complete RPMI 1640 medium for 60mins in an incubator. Following incubation, the cells are centrifuged and medium discarded and resuspended in new supplemented complete medium where they are ready for experimentation [180, 243].

5.2.10 Image flow cytometry

Dendritic cells conditioned with sAuNP or controls are stained intracellularly as described in chapter 2 for the assessment of particle uptake, lysosomes, endosomes, and nucleus. Cells are fixed for 10 minutes using 5% paraformaldehyde and then resuspended and washed with 0.5 saponin (Biolegend) twice before staining. Cells are incubated with fluorochrome conjugated monoclonal antibodies CD107a-PE-Vio615 (LAMP-1), CD71-PE-vio770 (Endosomes) (both from Biolegend) and DAPI (Sigma Aldrich) for 30minutes. AuNP conjugated with Hostasol green was used to look at uptake. After incubation cells are washed with 0.5 saponin, resuspended and fixed with 5% paraformaldehyde until analysis. Samples are analyzed using the MKII imagestream by Amnis and data is analyzed using IDEAS v6.2 Amnis software.

5.2.11 Statistical analysis

Statistical significance of the Data was obtained using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Therefore, paired t tests or ordinary one-way ANOVA was used when

comparing between two groups. Statistically significant p values were considered to be equal or lower than 0.05.

5.3 Results

5.3.1 sAuNPs do not activate immature dendritic cells (iDCs)

Initially we looked at sAuNPs ability modulate iDCs to understand if any of the combinations would stimulate cells to undergo maturation. We looked at surface phenotypic changes using CD86, MHC1 (HLA-ABC) and MHC2 (HLA-DR) as well as changes in IDO enzyme activity compared to AuNP conditioned cells. Data in figure 5.3 show no marked changes in CD86, MHC1 or MHC2 in iDC conditioned with sAuNPs. This data was consistent in IDO activity assessment of these cells where there is no increase in cellular enzyme activity (Fig.5.4). This indicates that both AuNPs and sAuNPs we used (Table 5.1) did not activate cells that were at a steady state. This data shows that these particles do not activate DCs which gives more flexibility in the addition of more immune stimulatory coatings to induce the required immune response.

sAuNP monosaccharide combinations		
100%Mannose	100% Galactose	100%Fucose
90% Mannose	90% Galactose	90% Fucose
10%Galactose	10% Fucose	10%Mannose
80% Mannose	80% Galactose	80% Fucose
20% Galactose	20% Fucose	20% Mannose
70% Mannose	70% Galactose	70% Fucose
30% Galactose	30% Fucose	30% Mannose
60% Mannose	60% Galactose	60% Fucose
40% Galactose	40% Fucose	40% Mannose
50% Mannose	50% Galactose	50% Fucose
50% Galactose	50% Fucose	50% Mannose
40% Mannose	40% Galactose	40% Fucose
60% Galactose	60% Fucose	60% Mannose
30% Mannose	30% Galactose	30% Fucose
70% Galactose	70% Fucose	70% Mannose
20% Mannose	20% Galactose	20% Fucose
80% Galactose	80% Fucose	80% Mannose
10% Mannose	10% Galactose	10% Fucose
90% Galactose	90% Fucose	90% Mannose

Table 5.1: Monosaccharide combinations used to coat AuNPs (sAuNP). Each of the AuNPs have been coated up to two monosaccharides from three different monosaccharides creating unique combinations.

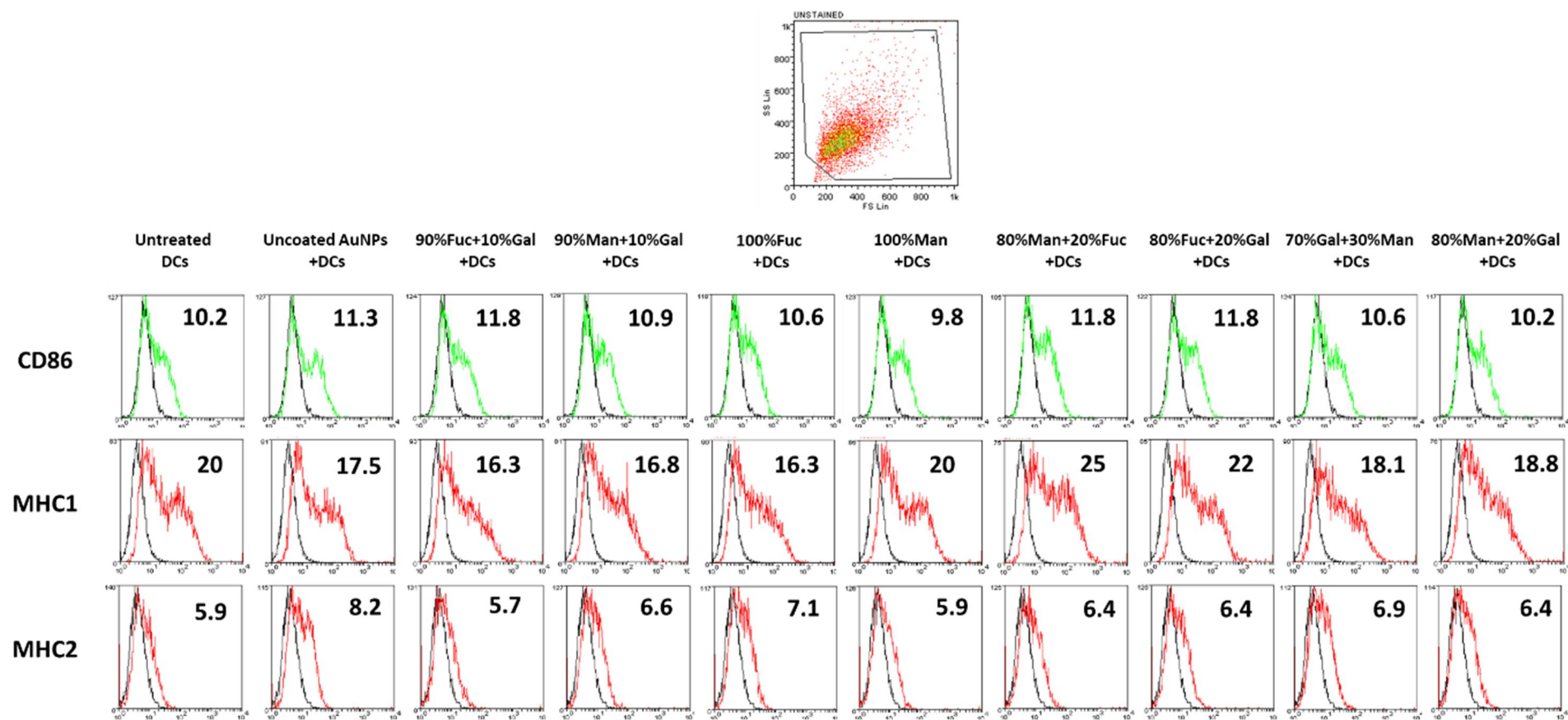


Figure 5.3: Flow cytometry phenotypic analysis of costimulatory markers CD86, MHC1 and MHC2 for sAuNP conditioned DCs. Data show no increase in the costimulatory markers suggesting no stimulatory effects of the sAuNP on iDCs. Data shown (MFI) is representative of 3 donors.

Determination of IDO Activity for sAuNPs as a surrogate for DC immunogenicity

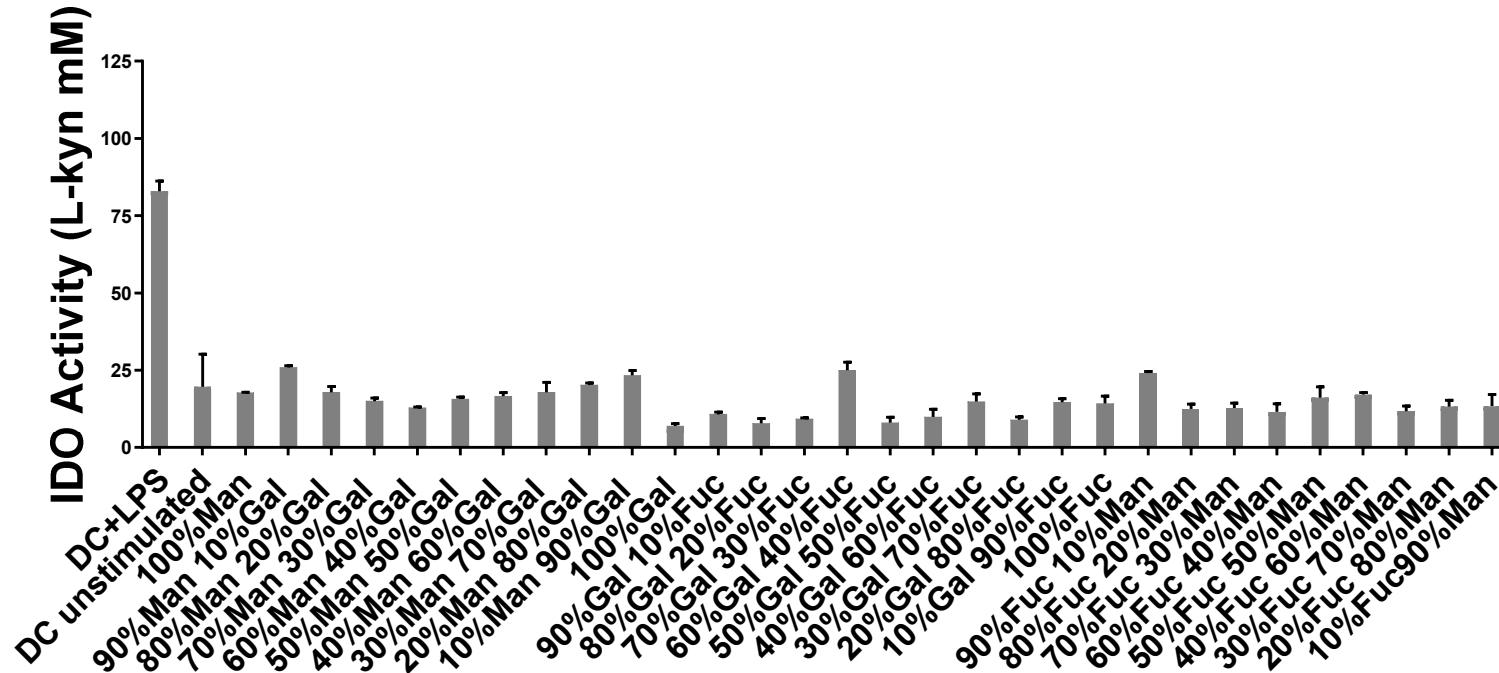


Figure 5.4: Estimation of IDO activity using kynurenine as a surrogate for DC immunogenicity after conditioning with sAuNPs. Data show no remarkable increase in IDO activity compared to unstimulated control. This suggests that sAuNPs did not have a stimulatory effect on DCs. Data shown are a mean $\pm$ SD of 3 separate donors.

5.3.2 Screening of sAuNPs uptake by dendritic cells

In order to understand uptake of our AuNPs and sAuNPs, we have used conjugated particles in which the fluorophore Hostasol Green is attached to the particles. Incubation of these particles with iDCs would allow uptake and washings would rid of excess particles in the medium leaving Hostasol content in the cell proportional to uptake of the particles.

Assessment of the uptake was done and compared against untreated iDCs as well as ICE incubated iDCs to ensure that particles do not adhere to cell surface instead of being internalized. Data shown in figure 5.5 illustrates uptake of sAuNPs combinations of mannose, galactose and fucose and uncoated particles. It can be clearly seen that some monosaccharide combinations promote the uptake of AuNPs more than others with having no uptake seen in ICE treated iDCs and most uptake seen in uncoated AuNPs. Combinations with highest and lowest uptake (Table 5.2) were selected to carry on additional experiments to understand these uptake patterns.

Highest uptake	Lowest uptake
100%Mannose	80% Mannose 20% Fucose
90% Mannose 10%Galactose	20% mannose 80% Fucose
80% Mannose 20% Galactose	60% Galactose 40% mannose
70% Mannose 30% Galactose	40% Galactose 60% Fucose
60% Mannose 40% Galactose	30% Galactose 70% Fucose
100%Fucose	20% Galactose 80% Fucose
90% Fucose 10% Galactose	

Table 5.2: Selected sAuNP conditions depending on highest and lowest uptake. These combinations of monosaccharide showed higher or lower uptake of sAuNPs by iDCs when compared to other combinations .

Dendritic cells uptake studies for sAuNP

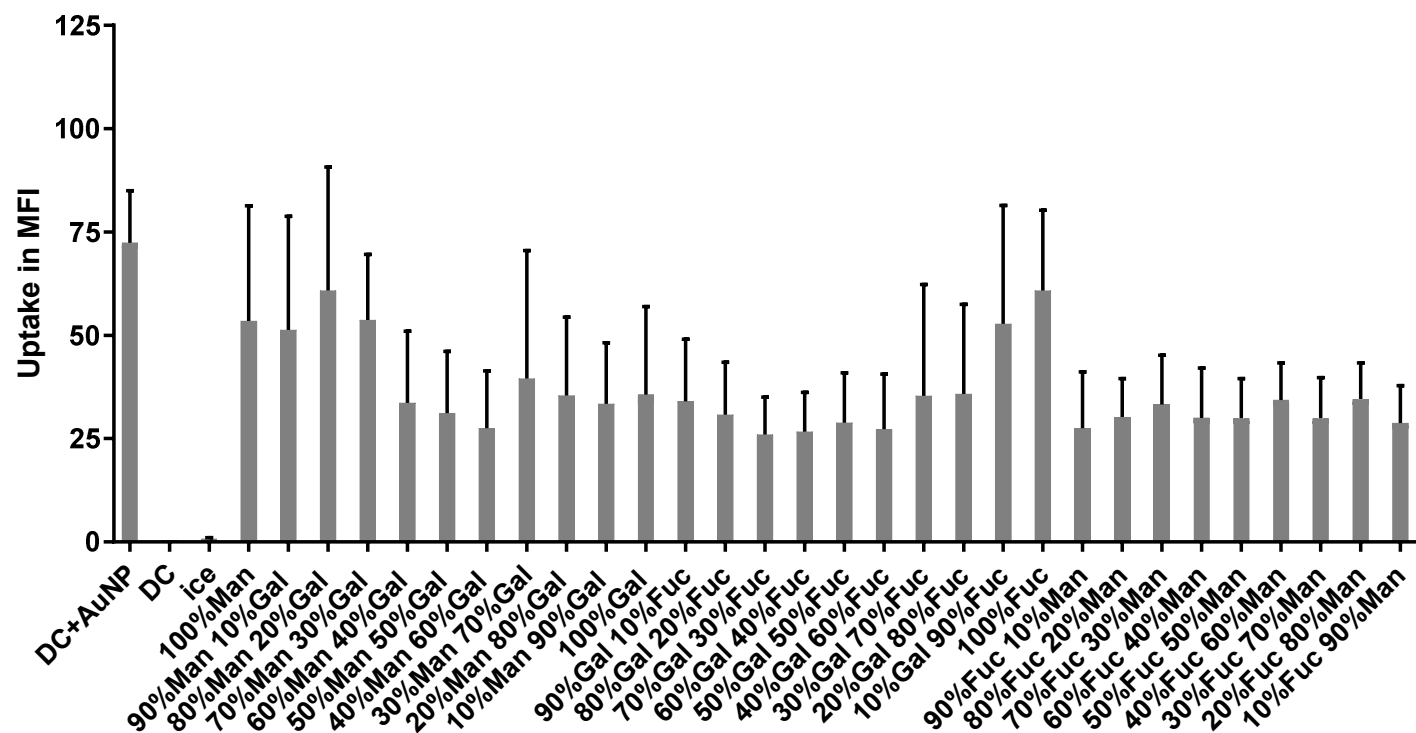


Figure 5.5: Flow cytometry data show uptake studies for sAuNP conditioned DCs. Different combinations have shown to promote uptake more than others where increase uptake is seen in uncoated AuNPs, 100%mannose, 90%mannose+10%galactose, 80%mannose+20%galactose, 70%mannose+30%galactose, 60%mannose+40%galactose, 100%fucose, 90%fucose+10%galactose. Data show are MFI $\pm$ SD of >3 separate donors.

5.3.3 Uptake of sAuNP mainly through receptor mediated mechanisms

To understand mode of cellular uptake of our sAuNPs, we decided to use Methyl- β -cyclodextrins (M β C). This substance enables the depletion of cholesterol from the cells membrane lipid rafts which are important for active clathrin mediated uptake while not having any implication on passive micropinocytosis. This would allow differentiation between passive and active internalization of sAuNPs which is important in understanding the intracellular route these particles would undertake. After the treatment with M β C, cells show significant reduction uptake of sAuNPs of different conditions (Fig.5.7a). Viability using Live/dead assay shows that M β C concentration used is not toxic to cells and that the reduction in uptake is not due to decrease in cell viability (Fig.5.7b). This indicates that the prominent mode of internalization of sAuNPs is via active phagocytosis.

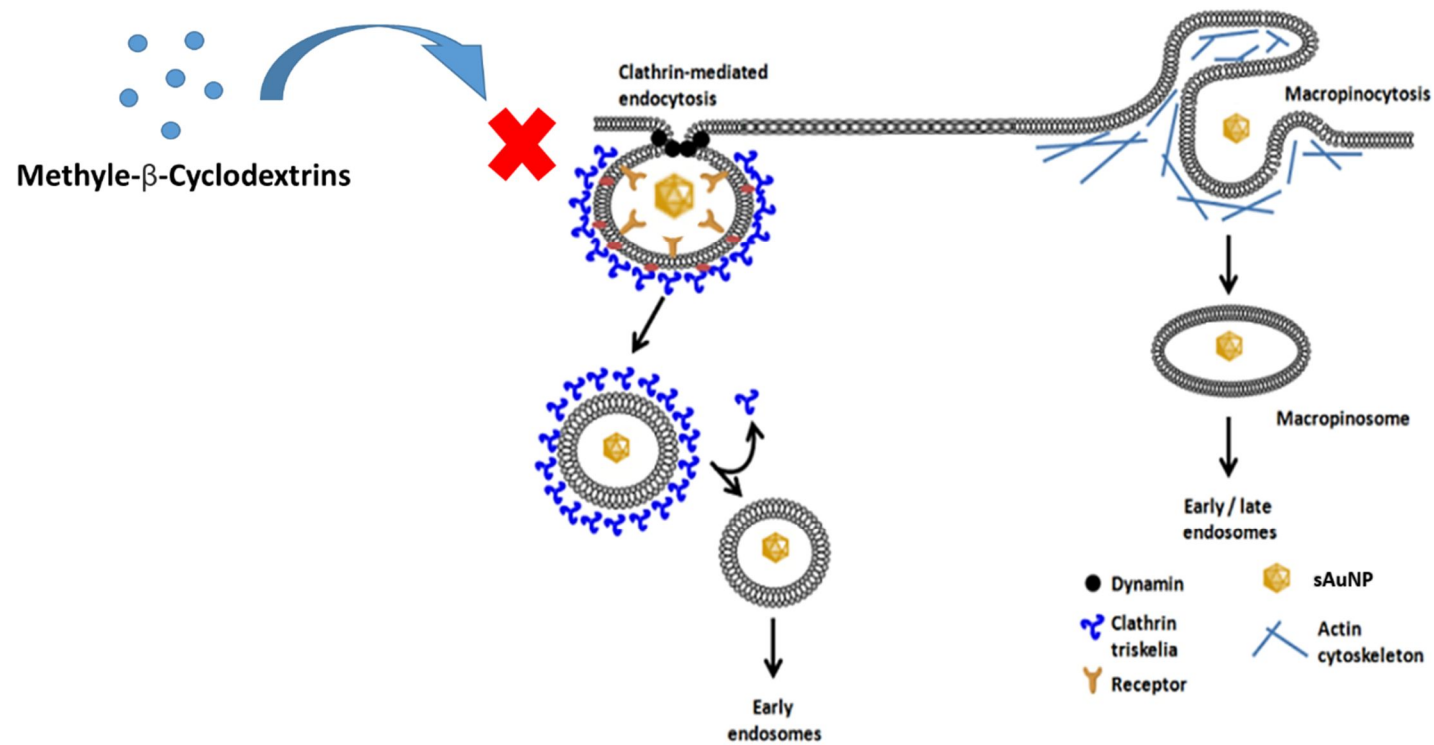
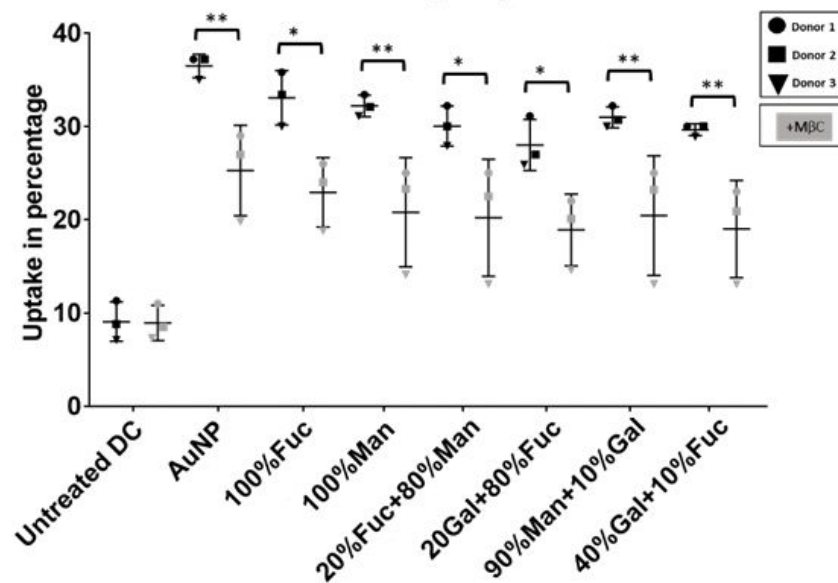


Figure 5.6: Depletion of cholesterol on DC membrane using Methyl-β-cyclodextrins. This enables the inhibition of the formation of lipid rafts and there for disabling active endocytosis but not passive endocytosis. This allows the understating of the means in which particles are internalized differentiating between active and passive endocytosis.

A

Determination of the uptake of sAuNP & AuNP after the depletion of the membrane cholesterol with Methyl- β -cyclodextrins



B

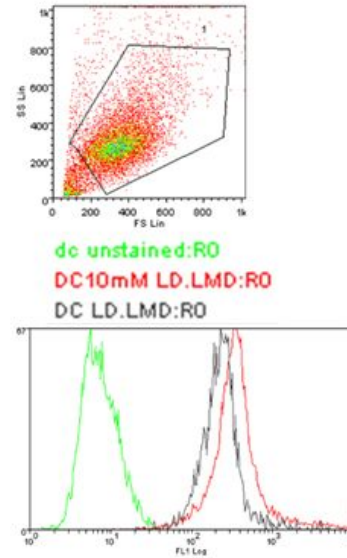


Figure 5.7: Uptake data obtained after the depletion of cholesterol on DC membrane using Methyl- β -cyclodextrins. A) There is a significant decrease in uptake of both coated and uncoated particles. This suggests that an active endocytosis is involved in the uptake of these particles however passive uptake is still involved as these particles are minute in size B) DCs viability using Live/dead flow cytometry stain suggest that the treatment with 10mM of Methyl- β -cyclodextrins did not affect DC viability. Data shown are a mean $\pm$ SD of three independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3.4 sAuNP modulates lysosomal and endosomal content in dendritic cells

To further understand our observations in active uptake of sAuNPs, we have looked at lysosomal and endosomal content in the selected conditions. Experiments were conducted at time points 1, 6, 24 hours to look at uptake in relation to lysosomal and endosomal content of cells. Figure 5.8 show increased uptake through time points reaching highest after 24 hours. The lysosomal and endosomal content show minimal content for both in cells in time points 1 through 6 hours where it only significantly increases after 24 hours. Looking at the 24hour time point in figure 5.9, there are sAuNPs conditioned iDCs that expressed higher lysosomal content seen in 100%fucose, 80%fucose+20%galactose and 100%mannose. These conditions also expressed high endosomal content except for 100%mannose.

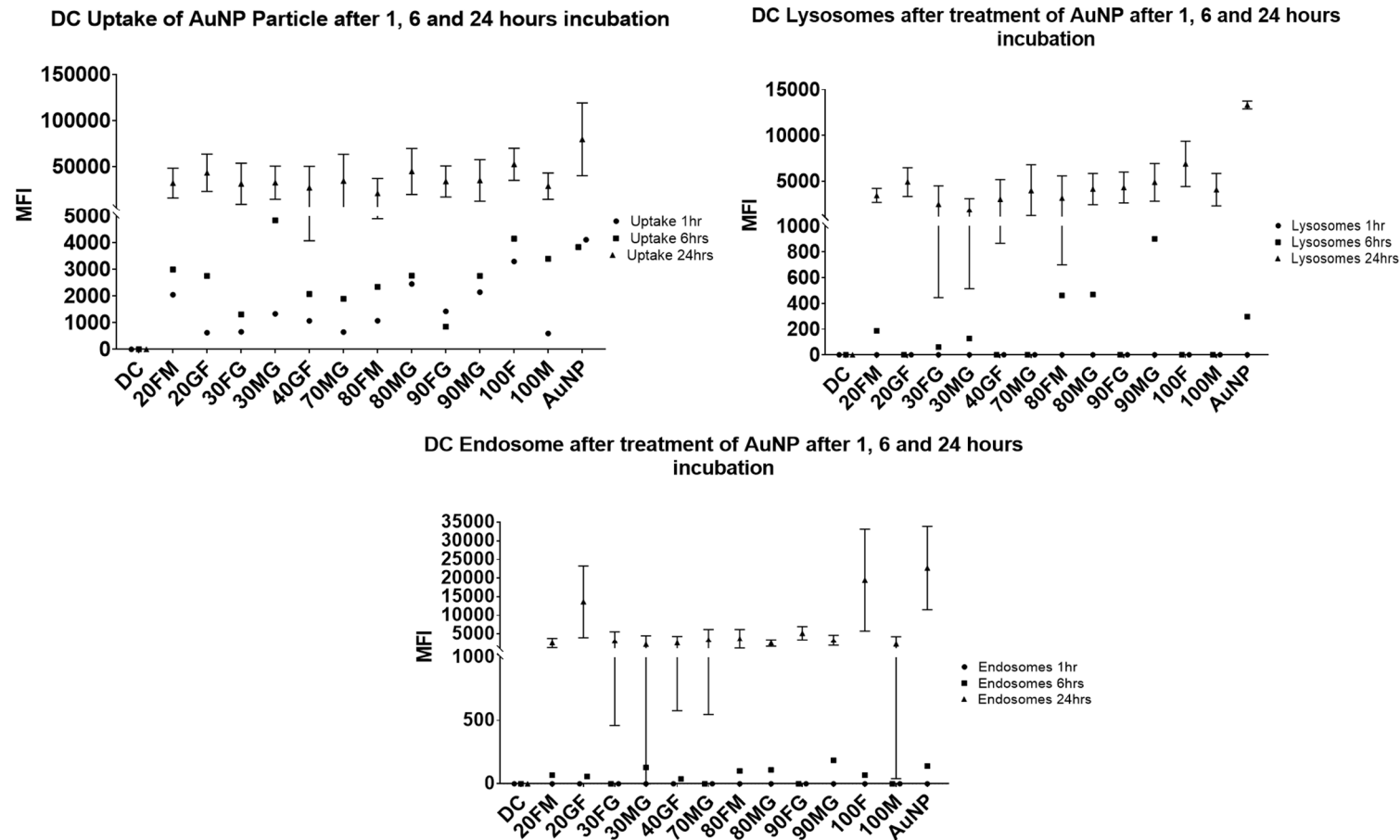


Figure 5.8: Time point experiments for sAuNP conditioned DC for the assessment of uptake, lysosomal and endosomal content. Data show similar uptake patterns for conditions in each time points. Increase in lysosomal and endosomal contents can be seen after 24 hours of culture. Data shown as mean $\pm$ SD for a single experiment for 1 and 6 hours time points and for >3 donors for 24 hours.

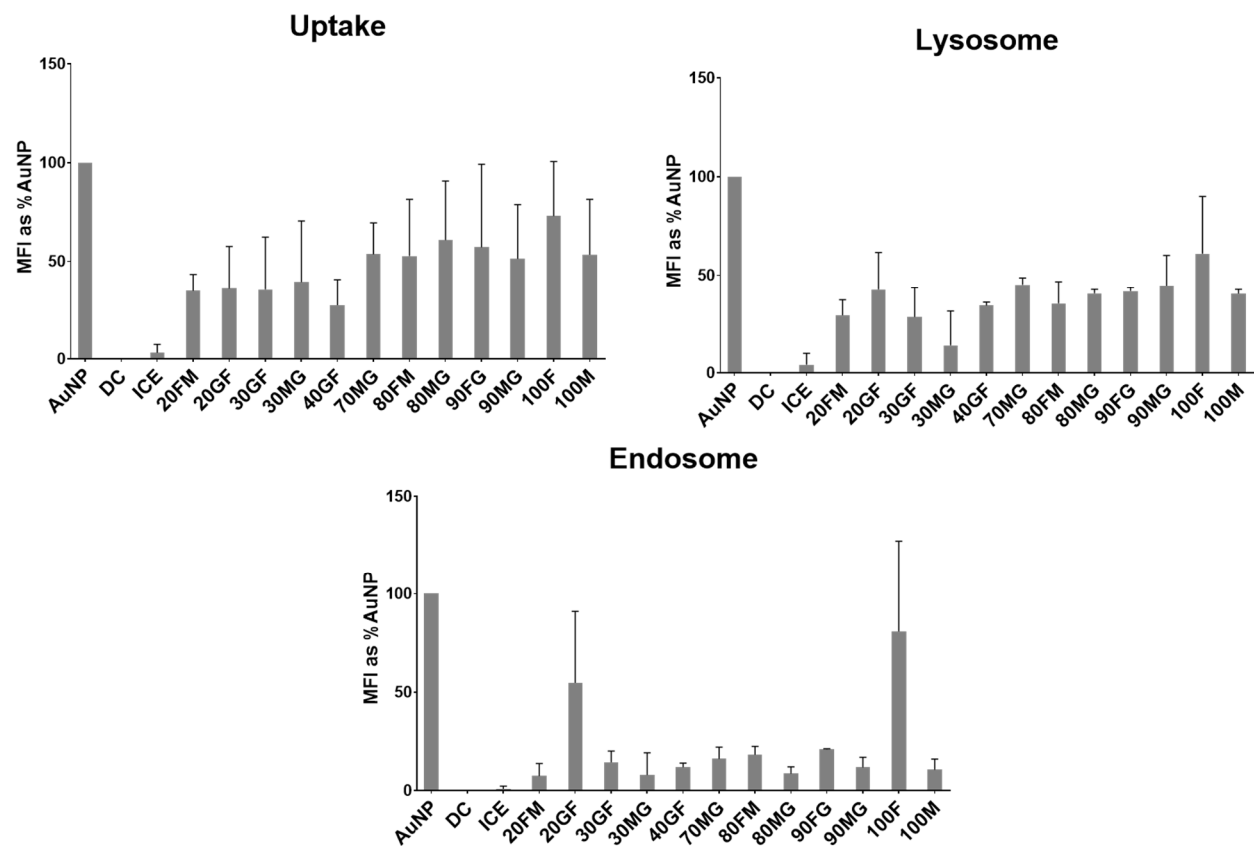


Figure 5.9: Flow cytometric analysis for uptake, lysosomal and endosomal contents for sAuNP conditioned DCs after 24 hour incubation .

High lysosomal content is seen in 100%fucose, 80%fucose+20%galactose and 100%mannose. These conditions also expressed high endosomal content except for 100%mannose. Data shown as mean $\pm$ SD for 3 donors.

5.3.5 Fucose and mannose sAuNPs promotes particle trafficking at lysosomes and endosomes respectively in dendritic cells

In order to link rapid uptake of the sAuNPs and antigen presentation routes through the lysosomal and endosomal contents (Fig.5.10), we have decided to look at the co-localization studies of sAuNPs in each of the lysosomal and endosomal compartments. Our aim is to find sAuNPs that are rapidly internalized and co-localized in lysosomal contents for antigen presentation. Data shown in figure 5.11 and 5.12 indicates that sAuNPs 100% fucose, 80%fucose+20%galactose and 70%galactose+30%mannose have significant increase in sAuNPs/lysosomal co-localization compared to uncoated AuNPs. These conditions show no significant increase in the sAuNPs/endosomal co-localization. On the other hand, 100%mannose show a significant increase in sAuNPs/endosomal co-localization while no significant increase in the sAuNPs/lysosomal co-localization compared to uncoated AuNPs.

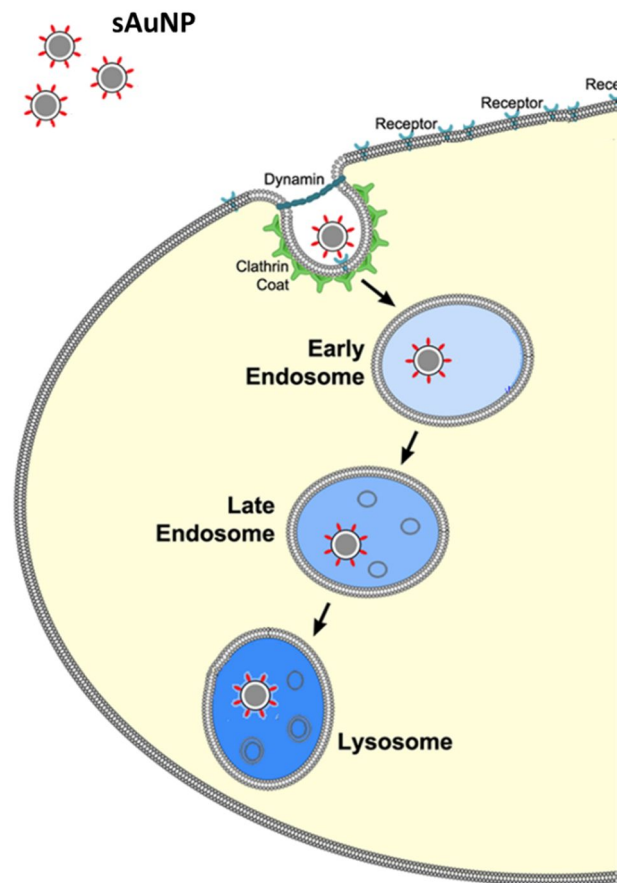


Figure 5.10: Illustrating uptake of sAuNP through active endocytosis by DCs. The internalized particles go through the invagination of the plasma membrane made by a protein coat in which an early endosome is created. The early endosome matures into the late endosome which then fuses with a lysosome. The contents are degraded by the lysosome enzymes and continue towards antigen presentation on MHC classes 1 and 2. Rapid delivery to lysosomes allows rapid antigen presentation of the particle's payloads.

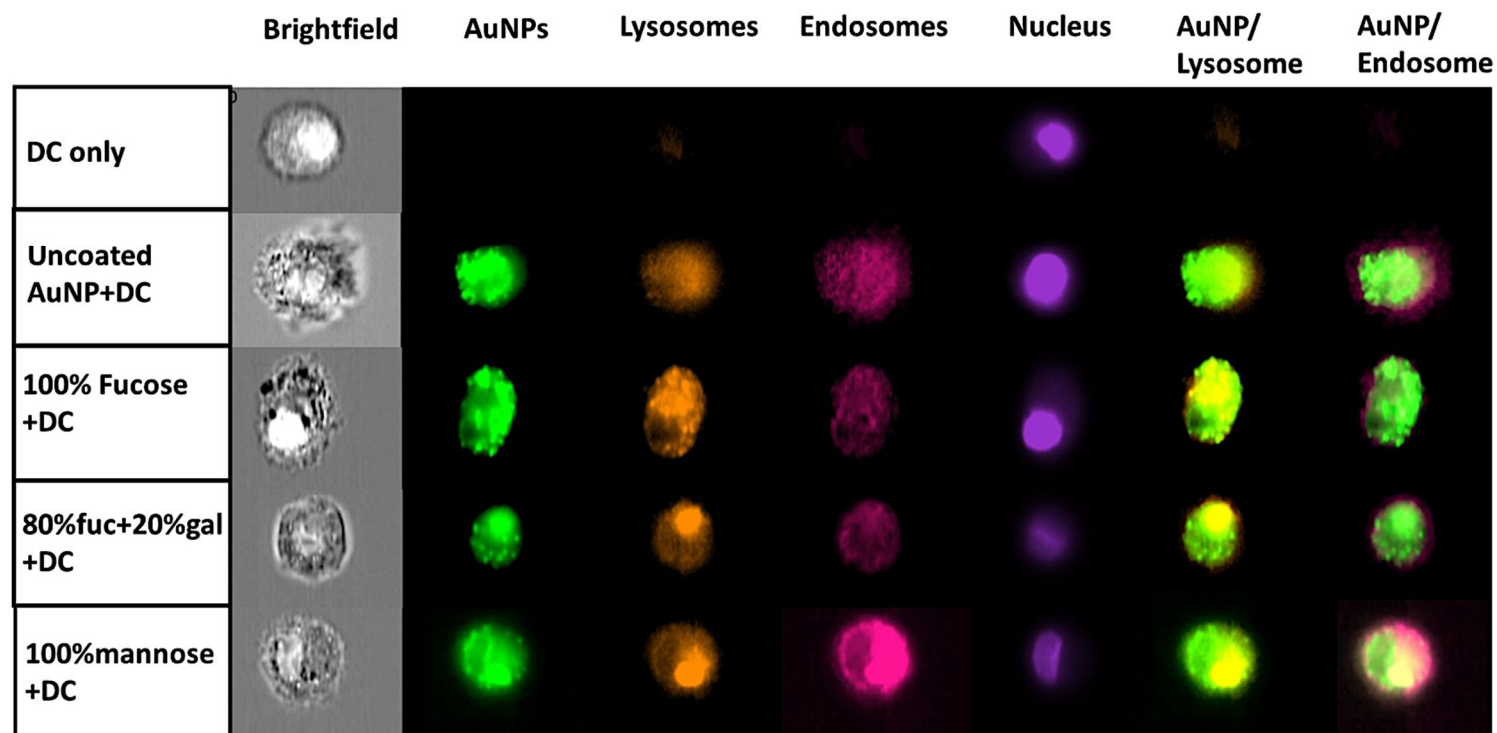


Figure 5.11: Images representing sAuNPs uptake Lysosomes (orange), endosomes (pink) and nucleus (purple) in DCs. Data confirms uptake by the conditions where most uptakes are seen in 100% fucose. It is also where most lysosomal co-localization can be noted followed by 80%fuc+20%gal. These conditions show reduced endosomal co-localization suggesting rapid particle delivery to lysosomal contents. On the other hand, 100%Mannose show reduced lysosomal co-localization and increased endosomal co-localization suggested delayed delivery of the particles to endosomes. Uncoated AuNPs show increase uptake but reduced co-localization for both endosomal and lysosomal contents. Data shown are of representative images of 60x of 3 donors.

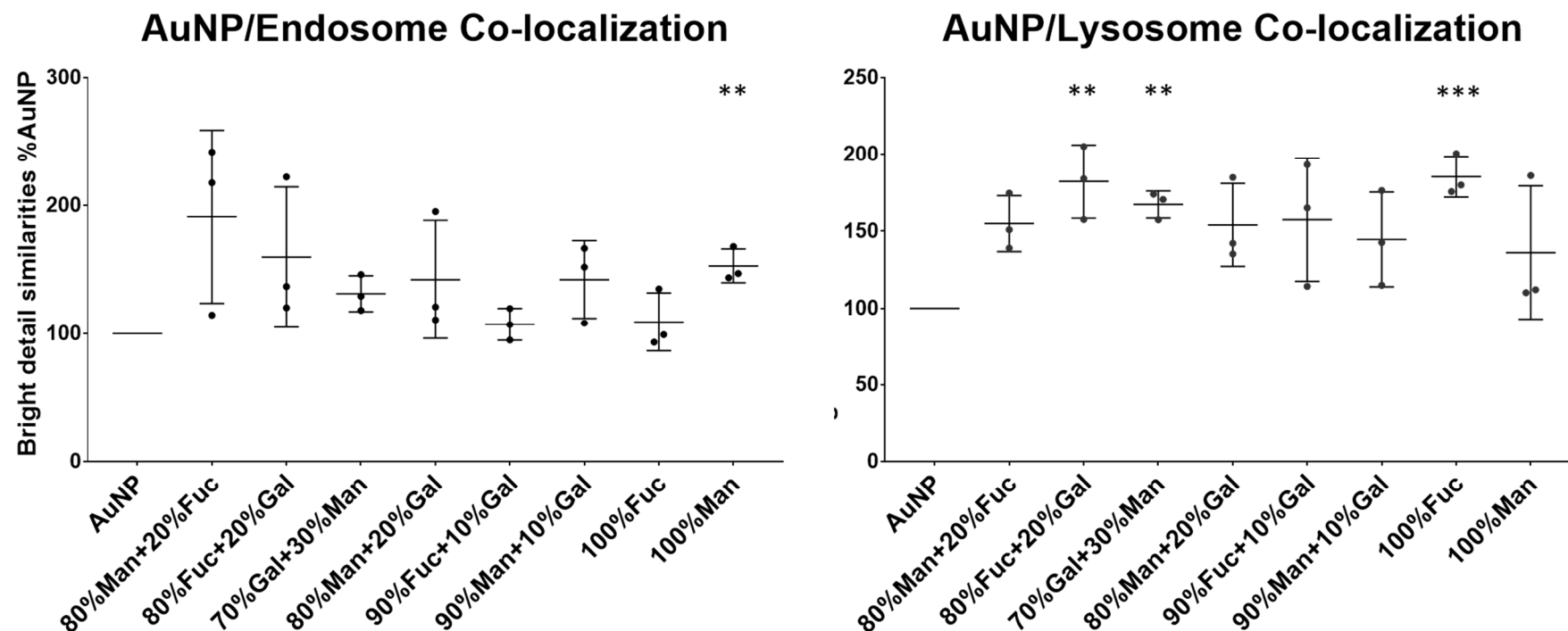


Figure 5.12: Illustrating Gold particle co-localization studies in lysosomal and endosomal contents in conditioned DCs. Data show that 100% fucose, 80%fucose+20%galactose and 70%gal+30%man have significant increase in sAuNPs/lysosomal co-localization compared to uncoated AuNPs. These conditions show no significant increase in the sAuNPs/endosomal co-localization. On the other hand, 100%mannose show a significant increase in sAuNPs/endosomal co-localization while no significant increase in the sAuNPs/lysosomal co-localization compared to uncoated AuNPs. Data shown are mean $\pm$ SD of three independent donors where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3.6 DCs conditioned with 100% Fucose and 20% galactose + 80% fucose but not 100% mannose sAuNPs promote T-cell proliferation

To understand the antigen presentation ability of conditioned DCs, we have used a DC-T cell co-culture and assessed T cell proliferation. CFSE proliferation data obtained (Fig. 5.13) show an increase in T cell proliferation in co-cultures conditioned with 100% fucose and 80% fucose + 20% galactose. Less proliferation can be seen in co-cultures conditioned with 100% mannose and uncoated AuNPs compared to unconditioned co-cultures. This data confirms observations in the increased co-localization of particles with lysosomes in which these conditions 100% fucose and 80% fucose + 20% galactose were able to cause proliferation of T cells while other conditions including 100% mannose and uncoated AuNPs did not promote T cell proliferation.

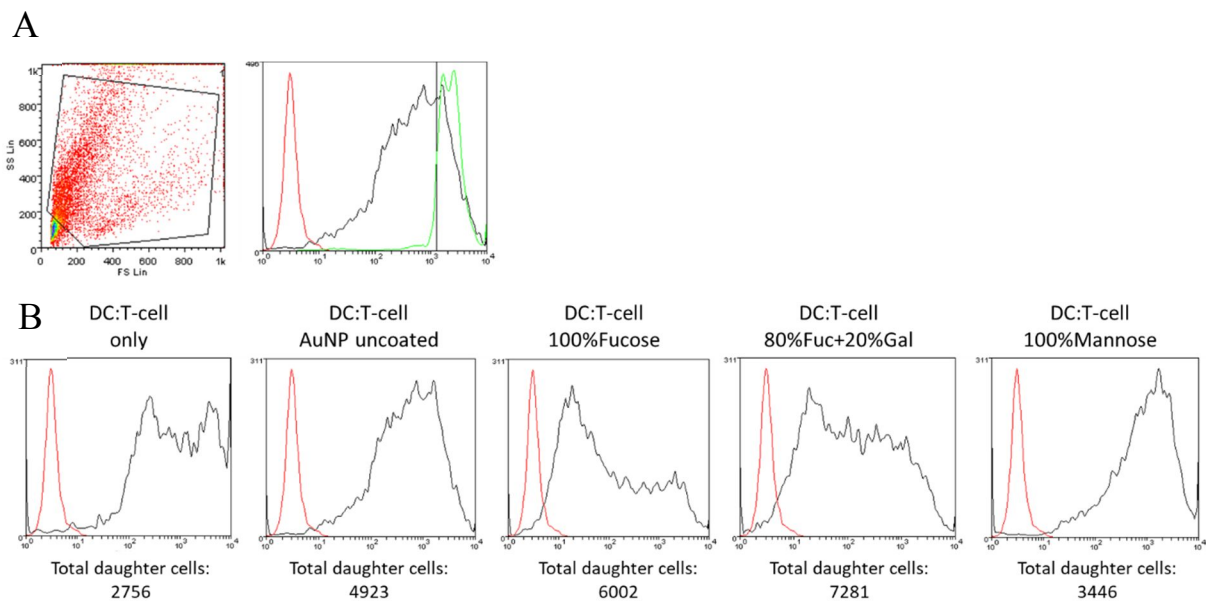


Figure 5.13: Flow cytometry data showing proliferation studies for DC:T cell co-culture after conditioning DCs with sAuNPs. Increase in daughter cells suggests increased proliferation in conditions 100%fucose and 80%fuc+20%gal compared to DC:T cell only and other conditions. This follows previous data where these conditions show rapid delivery to lysosomes and rapid antigen presentation. The same concept is for 100%mannose where there is a low proliferation profile going in line increase in endosomal co-localization of the particles and slower antigen presentation.

5.4 Discussions

AuNPs have been studied as platforms for drug delivery and immunomodulation of DCs. Optimization of AuNPs coatings not only allow targeted uptake of these particles but also enhancing uptake and guiding its mechanisms. Using AuNPs to deliver tumor antigens to DCs as a mean of anti-tumor vaccination has shown great progress [266, 267]. Coating AuNPs with monosaccharides enable targeting different CTLs on DCs surfaces and once uptake would go through antigen presentation pathway. Different monosaccharides would interact with different surface CTLs and a combination of monosaccharides would enhance recognition and uptake of a certain particle. Monosaccharides coating are not well studied as coatings for drug delivery but rather as ligands for guiding uptake only using their absolute forms. In this context we have set out to study how different monosaccharide combinations are able to modulate AuNPs uptake by DCs and the pathway these particles go through. This would provide information on combinations that have highest uptake and rapid localization towards lysosomal compartments which would prove ideal coatings for antigen delivery to DCs.

Initially we looked at the sAuNPs effect on DCs through understanding the phenotype and IDO enzymatic activity of these cells after conditioning with different sAuNPs combinations. Phenotypic markers CD86, MCH1 and MHC2 are known to be upregulated in stimulated DCs. Data show no changes in these markers suggesting no stimulation effects by sAuNPs on DCs. This is consistent with IDO enzyme reading where no changes are observed in conditioned DCs. Studies in this prospective have been contradicting where some have shown that AuNPs cause DC stimulation and maturation [260, 268] while others have opposing findings [269, 270]. This is thought to be due to the combination of surface charge

and size of particles where they are able to cluster and bind to CTLs activating DCs or not [271, 272].

The key advantage of coating AuNPs with monosaccharides is to promote uptake and potentially target AuNPs delivery into different cellular compartments. Mannose is a known ligand for DC-SIGN and MR and has been widely used in soluble form and as coating for NPs to target these CTLs. However, other monosaccharides have not been widely used as coatings and combinations of these monosaccharides are not well studied. In the next set of experiments, we decided to look at combinations of monosaccharides to understand their ability in promoting uptake and enhancing the diffusion of AuNPs into DCs. Data obtained show different uptake patterns suggesting that some monosaccharide combinations enhance uptake while others reduce uptake comparing to uncoated AuNPs. Combinations consisting of mannose and galactose show direct correlation between uptake and the content of mannose where greater uptake can be correlated in combinations with greater mannose content. This is not surprising as most intracellular pathogens bind to CTLs using mannose as a ligand. Similarly, fucose and galactose combinations where the correlation of uptake is directly proportional increasing with increase in the fucose content. This indicates that galactose incorporation to the combinations decrease uptake. This can be explained that CTLs responsible for uptake of fucose and mannose both MR and DC-SIGN are much more expressed up to 10 times more in DCs than MGL which is responsible for galactose uptake [273]. This also shows why pathogens have evolved to express ligands for these receptors such as mannose in *Mycobacterium* or fucose in *H.pylori* or both such as *Faciola hepatica* to modulate DCs response and uptake mechanisms. Pathogens coated with galactose are mostly fungi and parasites or aberrated glycosylation patterns in tumours Tn antigens where they are not engulfed but rather stimulate alternate immune responses. Interestingly, combinations consisting of both fucose and mannose show low uptake and are the least sAuNP

combinations to be internalized by DCs. This does not indicate a decrease in uptake but rather combinations that do not promote enhanced uptake by DCs. Studies have shown that *Faciola hepatica* antigens contain mainly mannose and fucose and interact with DC-SIGN and MR where they are internalized. Other studies show fucose functionalized AuNPs promote uptake and internalization by DC cell lines. However, none of these studies compared uptake between different concentrations of fucose or mannose and how that would influence internalization.

To better understand our data, we decided to look at the influence of active and passive endocytosis for both AuNPs and sAuNPs. This is done through the depletion of cholesterol from the lipid bilayer using M β C and reducing raft formation and active endocytosis while not effecting passive endocytosis. This would provide information on the pathway these particles would be undertaking. Data show a decrease in uptake for sAuNPs and AuNPs in the M β C treated cells compared to untreated cells. This indicates that both monosaccharides coated and uncoated AuNPs are interacting with CTLs. This is as mentioned previously that AuNPs are able to through surface charges aggregate and cause CTL activation and internalization. In regard to sAuNPs, it is no surprising that monosaccharides are interacting with CTLs which forms a big part of the internalization. This is said, this also shows that there is another method of uptake which is the passive macropinocytosis which small particles gain access to the cells with no interaction with surface CTLs.

Our findings suggested interaction of the monosaccharide coatings with surface receptors in which internalization was through an active endocytosis. In this we have identified a selection of high uptake and low uptake conditions in which we decided to look at the fate of the sAuNPs through understating the endosomal and lysosomal content. The selected

combinations with highest uptake [100%mannose, 90%mannose+10%galactose, 80%mannose+20%galactose, 70%mannose+30%galactose, 60%mannose+40%galactose, 100%fucose, 90%fucose+10%galactose] and of lowest uptake [40%mannose+60%galactose, 40%galactose+60%fucose, 30%galactose+70%fucose, 20%galactose+80%fucose, 80%fucose+20%mannose, 80%mannose+20%fucose]. It is known that active uptake leads to degradation through the lysosomal pathway and presentation of the antigens on MHC molecules. Flow cytometry data for the lysosomal and endosomal content for these conditions show that the highest contents are for uncoated AuNPs which is followed by 100%fucose. On the other hand, condition 80%fucose+20%galactose had increase in endosomal content with no increase in lysosomal content compared to other sAuNPs. To supplement this data, we have analysed images of DC to establish co-localization of the particles in the endosomal or lysosomal contents. Data show and despite increase in lysosomal and endosomal content of uncoated AuNPs that there is much less co-localization of these particles with both endosomal and lysosomal contents. Monosaccharide coated AuNPs 100%fucose and 80%fucose+20%galactose have significant increase in co-localization of particles with the lysosomal content compared to uncoated AuNPs. On the other hand, 100%mannose show increase in co-localization of particles with endosomal content. This indicates rapid uptake and delivery of particles to lysosomes through the coating with 100%fucose while much less uptake can be achieved through incorporation of galactose but rapid lysosomal delivery. Mannose shows increased uptake but less efficient lysosomal delivery and rather more particles are trapped in the endosomal phase. This shows that fucose coatings enhance uptake and rapid delivery for payloads for presentation through MHC complex while mannose is more suitable for coatings to escape lysosomal coatings and carry payloads directed for nucleus for mRNA silencing.

Finally, we have decided to look at the interaction of sAuNPs treated DCs with naïve T cells. This would provide valuable information regarding the ability of these conditioned DCs to activate T cells establishing the facts related to rapid payload delivery and presentation on MHC complex. Data show that conditions 100%fucose and 80%fucose+20%galactose were able to induce naïve T cell proliferation greater than controls. On the other hand, uncoated AuNPs and 100%mannose were not able to induce T cell proliferation. This goes hand in hand without findings in which rapid co-localization with lysosomes would mean increased presentation by these cells and thus increasing MHC complex expression and activating naïve T cells. Other conditions that had low lysosomal co-localization or high endosomal co-localization had no ability to activate naïve T cells.

5.5 Conclusions

In conclusion we were able to show how monosaccharide coatings are able to enhance uptake and modulate trafficking of payloads into DCs. Identified monosaccharide coatings show rapid lysosomal delivery with increase in fucose content while increase in mannose contents promote particles to stay in the endosomal phase. These findings provide knowledge in use of monosaccharides as coatings for rapid antigen delivery and presentation in DCs using fucose combinations can be valuable for vaccinations which aids in the fight against tumors.

Similarly, the use of mannose combinations coatings to delay payloads in the endosomal phase can be used in therapies requiring endosomal escape of payloads and delivery into the inner cellular compartments such as mRNA silencing therapies.

Chapter 6:

General discussion

The immune system has a repertoire of immune cells at its disposal to protect the host against different threats and invaders [274]. APC carry the role of mediators between the innate and adaptive immune responses to trigger a more efficient, specific and potent response and eliminate threats [275]. Dendritic cells are professional antigen presenting cells that are capable of antigen capture, processing and presentation to the adaptive immune cells [276]. This ability has made these cells the centre of attention for research as targets for vaccinations, drug delivery and immune modulation [277]. Different methods have been used for modulation and payload delivery for DCs. These methods have in common the interaction between the mediators and surface receptors mainly CTLs on DCs [278]. Monosaccharides have been known as a ligand for CTLs and are means in which pathogens and tumours interact with host cells [279]. Several pathogens have evolved to target certain lectins to modulate or even gain access into intracellular compartment and causing their pathology [280]. Tumour cells have been known to express aberrant glycosylation patterns in which able to interact with immune cells and as part modulate these cells into tumours favour as well as promote tumour metastasis [281]. Research have focused and proved polysaccharides and glycoconjugates potentials in driving and modulating the immune responses [282]. Monosaccharides immunomodulatory potential is poorly understood and the use as ligand drug delivery remains in its infancy In this context we investigated different monosaccharides combinations in immobilized, soluble and polymer attached with a goal to unveil monosaccharide combinations that possess immunomodulatory in immobilized form and promote uptake and rapid antigen presentation in polymer attached form. The main hypothesis remains that monosaccharides are able to interact with surface lectins modulating DC behaviour. Successfully identifying modulatory monosaccharide combinations would provide novel tools for the development of anti-tumour therapies, wound healing and regeneration as well as autoimmune therapies.

Previous studies have shown the immunomodulatory properties of glycoconjugates coating pathogens and tumours on DC behaviour [283, 284]. This is through shifting DCs response from inflammatory to tolerogenic which in turn suppresses the immune system [285]. ManLam is a glycoconjugates coating Mycobacterium terminated in mannose in which reacts with CTLs on DCs. This causes the cells to increase in IL-10 and lower IL-12 production leading to suppression of the immune system [286]. The same concept is utilized by *Helicobacter pylori* but the glycoconjugates are terminated with fucose causing DC tolerogenicity and a humoral response which does not clear the infection and instead causes persistent inflammation [287]. Malignant tumour cells tend to over express Lewis X antigen in which is terminated with galactose interacting with MGL on DCs causing suppression of inflammation [288]. When looking at our data this was the case when using only the monosaccharides in immobilized forms. Our mannose, galactose and fucose combinations were able to modulate DC phenotype. This was seen through the reduction of costimulatory markers CD40 and increase in tolerogenic CD274 expressions for LPS challenged DCs. These DC have attained a mature phenotype as confirmed by increased levels of CD86. The ability of CD40 to engage CD154 on T cells and initiate a pro-inflammatory response increasing proliferation and differentiation of T cells towards Th1. Instead the decrease in CD40 accompanied with increase in CD274 promote a more tolerogenic and humoral T cell response. This is consistent with how pathogens modulate the immune system in their favour. Clare M. Hamilton et al showed that *Faciola hepatica* Tegumental antigen -a glycoprotein that is fucosylated- modulated CD40 expression in TLR activated DC [289]. Moreover, comparing the immobilized monosaccharides with their soluble counterpart shows that cells behave differently to the same substance depending on the mechanisms of presentation. Soluble monosaccharides did not have any modulatory effect on both immature and mature DCs while immobilized monosaccharides were able to modulate DCs phenotype. Soluble monosaccharides including mannose have been used as competitors

to block CTLs in studies indicating that they would have no effect otherwise on cell behaviour [290].

Indoleamine 2,3-dioxygenase is an enzyme known to be involved in immune cell activation and stimulation [291]. We have shown in chapter 3 that this enzyme has a direct relationship to DCs inflammatory condition. The data obtained for the immobilized monosaccharides conditioned DCs show significant reductions in IDO activity compared to untreated controls. This is consistent with the phenotypic changes previously observed. A reduction in CD40, increase in CD274 and reduction in IDO activity would suggest a tolerogenic DCs phenotype. Similar findings were observed by Shih Ling et al where inhibition of IDO activity suppressed DCs inflammatory responses to LPS [292].

To confirm the presence of tolerogenic DCs we looked at the cytokine profile of the conditioned DCs. Cytokine profiling have shown a significant increase in anti-inflammatory IL-10 and a decrease in IL-12 in conditioned DCs. The data is confirmatory for the significant decrease in IDO activity patterns which represents DCs immunogenicity. This suggests that monosaccharides have a potential in significantly reducing inflammation for LPS challenged DCs through modulating the TLR-CTL crosstalk. These findings provide enough evidence that our HIT immobilized monosaccharides possess immunomodulatory potential suppressing DCs inflammatory responses to TLR4 activation. Research have shown the link between CTL and TLR and how pathogens binding through these receptors can modulate the immune system [75].

The biological relevance of our monosaccharide conditioned DCs and the downstream effects of the immobilized monosaccharides depend on the ability to modulate the adaptive immune response. This is translated into cell to cell communication between conditioned DCs and naïve T cells. This *in vitro* co culture approach have been used to understand *in vivo* cell to cell

interactions and determine immune response outcomes [293]. Data obtained from our co-culture of monosaccharide conditioned DCs and naïve T cells showed remarkable changes in cytokine profile compared to control. Naïve T cells have shown an increase in anti-inflammatory IL-10 in HIT conditions indicating presence of tolerogenic DCs that drive such response from T cells. Interestingly HIT conditions where galactose is incorporated show a significant increase in IL-17 while a significant decrease in IL-17 in one of our HIT conditions consisting of mannose and galactose. These findings come consistent with research focusing on organisms displaying these monosaccharides and the outcome of the immune reaction. Organisms such as mycobacterium display proteins glycosylated with mannose and others such as *faciola hepatica* displaying both mannose and fucose have been shown to increase IL-10 production in T cells through modulating DCs into tolerogenic phenotype [294-296]. A study has observed an increase in IL-17 production by T cells through co-culture with engineered mouse tolerogenic DCs [238]. T cells are known for their plasticity and would undergo change in the microenvironment shift from one phenotype to another or exhibit more than one phenotype [217]. In this case we hypothesised presence of a population of T cells expressing both T-reg and Th17 genes. To confirm our findings, we have performed Real time PCR on these T cells for the genes FOXP3 and ROR γ t which are expressed in T-regs and Th17 respectively. PCR data show increase in FOXP3 and ROR γ t for galactose HIT conditions while reduction in ROR γ t in HIT condition consisting of mannose and galactose. This confirms previous cytokine data findings and provides more evidence on the presence of these populations in our T cells. Research have described regulatory T cells with the capacity to produce IL-17 to be highly immune suppressive and are believed to be related to tumour survival [297]. As discussed previously, some malignant cancer cells are known to express aberrant Lewis X antigen which is galactosylated which in turn interact with DCs CTLs and cause immune suppression [298].

This is consistent with our findings with galactose HITs which is a tool that can be harnessed in immune therapies against inflammatory conditions.

On the other hand, the presence of mannose with galactose in the combination have shown a decrease both in IL-17 production and ROR γ t expression while maintaining increased levels of IL-10 and FOXP3. This condition was further assessed by using an *in vitro* allergy model to understand the conditioned cells potency in combating inflammation. Data show that galactose HIT condition behave similarly in the allergy module with increased IL-10 and unchanged levels of IL-17 showing consistency of the outcome. The mannose and galactose HIT condition also were consistent with previous findings in reduction of IL-17 in the allergy induced *in vitro* module while IL-10 levels remain increased. These outcome show potency of the treated cells and influence of the immobilized monosaccharides in which provide novel therapeutic tools for management of inflammatory conditions.

The use of nanoparticles in drug delivery and cell modulation have been showing promising outcomes for the development of personalized medicine [299]. Nanoparticles can be synthesised from virtually any material, easily shaped and functionalized or loaded with substances or drugs [300]. This provides a wide range of choices each is more efficient to address the wide range of diseases and disorders [301]. Gold nanoparticles have been praised for its stability both physically and chemically, low toxicity rate and being inert thus having no biological effect on cells and tissues [302]. This enabled the use of AuNPs as mediums for drug delivery, imaging tools in radiology and immune modulators through functionalization of immunogenic compounds or antibodies [303, 304]. This being said, studies have rarely looked at monosaccharides roles as coatings of polymers. Blanca Arnáiz et al have used AuNPs coated with HIV gp120 mannnsides to block DC-SIGN on DCs and inhibit trans-infection of HIV to T cells *in vitro* [268]. Another study have looked at α -fucosylamide coated AuNPs as means of targeted delivery for DCs [269]. However. To optimize and

understand monosaccharides potential as immune modulators or enhancing uptake and presentation, we need to look at larger array of monosaccharide combinations.

Initial experiments were to look at immunomodulatory effects of the monosaccharide attached AuNP on DCs. This has not been addressed rather AuNPs of different shapes, charges and sizes were assessed for immunomodulatory properties. Our data show that our 16nm sAuNPs did not stimulate or activate DCs. This was obtained through assessment of costimulatory molecules CD86, MHCI and MHCII expression on conditioned DCs. This was similar to how soluble and immobilized monosaccharides where there was no activation or stimulation effects on DCs.

Next, we looked at uptake of sAuNP through tagging with the fluorochrome Hostasol green. Interestingly, uptake varied between monosaccharide combinations showing a pattern where increase in uptake is proportional to increase in fucose or mannose content but not galactose. This is believed to be due to higher expression of mannose and fucose CTLs on iDCs such as DC-SIGN and MR rather than MGL. This is consistent with literature using mannose or fucose functionalized AuNPs for enhanced uptake and DC targeting. This also in line with microorganisms and parasites expression of mannose and fucose but not galactose to target immune cells. Intriguingly was our finding that uncoated AuNPs had greater uptake than most sAuNP. Due to the size we believe that passive uptake through micropinocytosis was the mean of uptake for uncoated AuNP while sAuNPs would engage CTLs and active uptake. Through cholesterol inhibition using M β C, we stopped formation of lipid rafts and active uptake leaving passive uptake functional. Conditioned DC showed significance decrease in uptake for both sAuNP and uncoated AuNPs. This indicates involvement of active uptake and CTL engagement for sAuNP. Regarding uncoated AuNP it is believed that uncoated particles in culture medium would adsorb protein forming a “protein corona” that would react to cell surface receptors and result in endocytosis [305]. HIT conditions were selected based on top

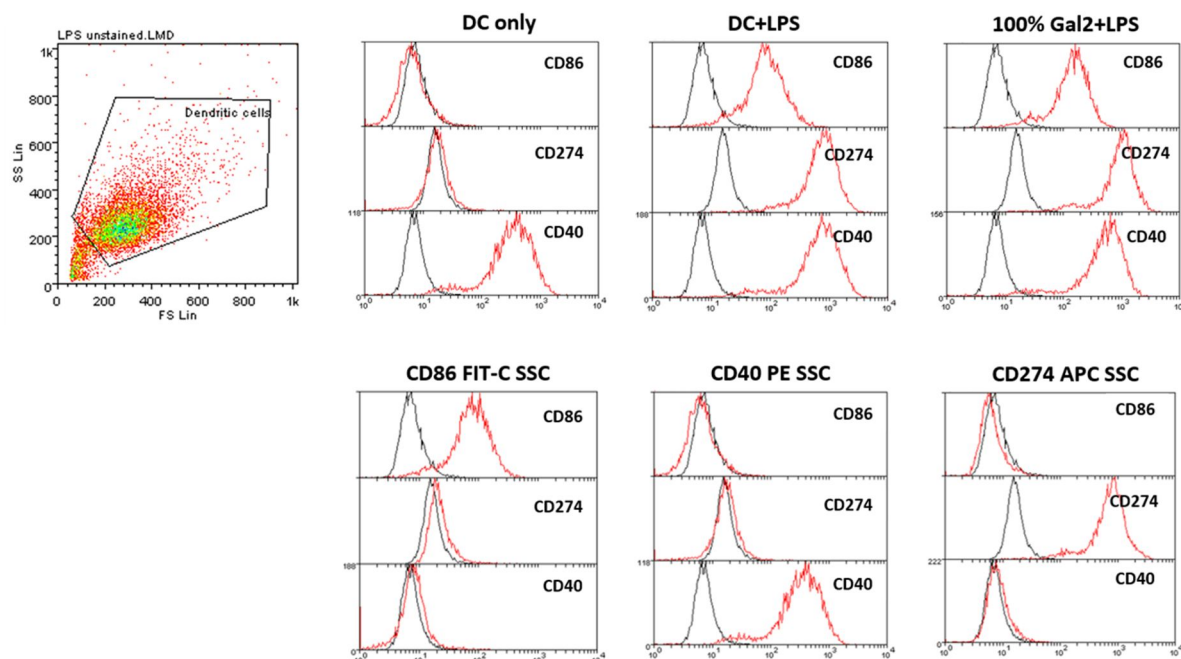
five highest and lowest uptake monosaccharides to carry additional experiments looking into the fate of the particles.

Active uptake of particles is known to go through the pathway towards presentation on MHC proteins on DC surface [306, 307]. However, we aimed at understanding influence of monosaccharide ligation to CTLs on the lysosomal and endosomal content and localization during 24hrs. This would provide evidence on the how rapid antigen presentation is. Data show increased lysosomal content for fucose consisting HITs. Co-localization data through imaging indicated increased lysosomal/sAuNP co-localization for fucose HITs while uncoated AuNPs had lowest co-localization for lysosomal/AuNP and endosomal/AuNP. Interestingly, 100%mannose showed increase in endosomal/AuNP co-localization. This translated into enhanced uptake and rapid delivery to lysosomes by fucose sAuNPs while much slower delivery having sAuNP remained in endosomes with 100%mannose. This provides new insights into using sAuNPs for rapid antigen delivery to lysosomes for vaccination using fucoses while enhanced uptake and slower delivery is suitable for using siRNA therapies to escape from endosomes and reach target in the nucleus.

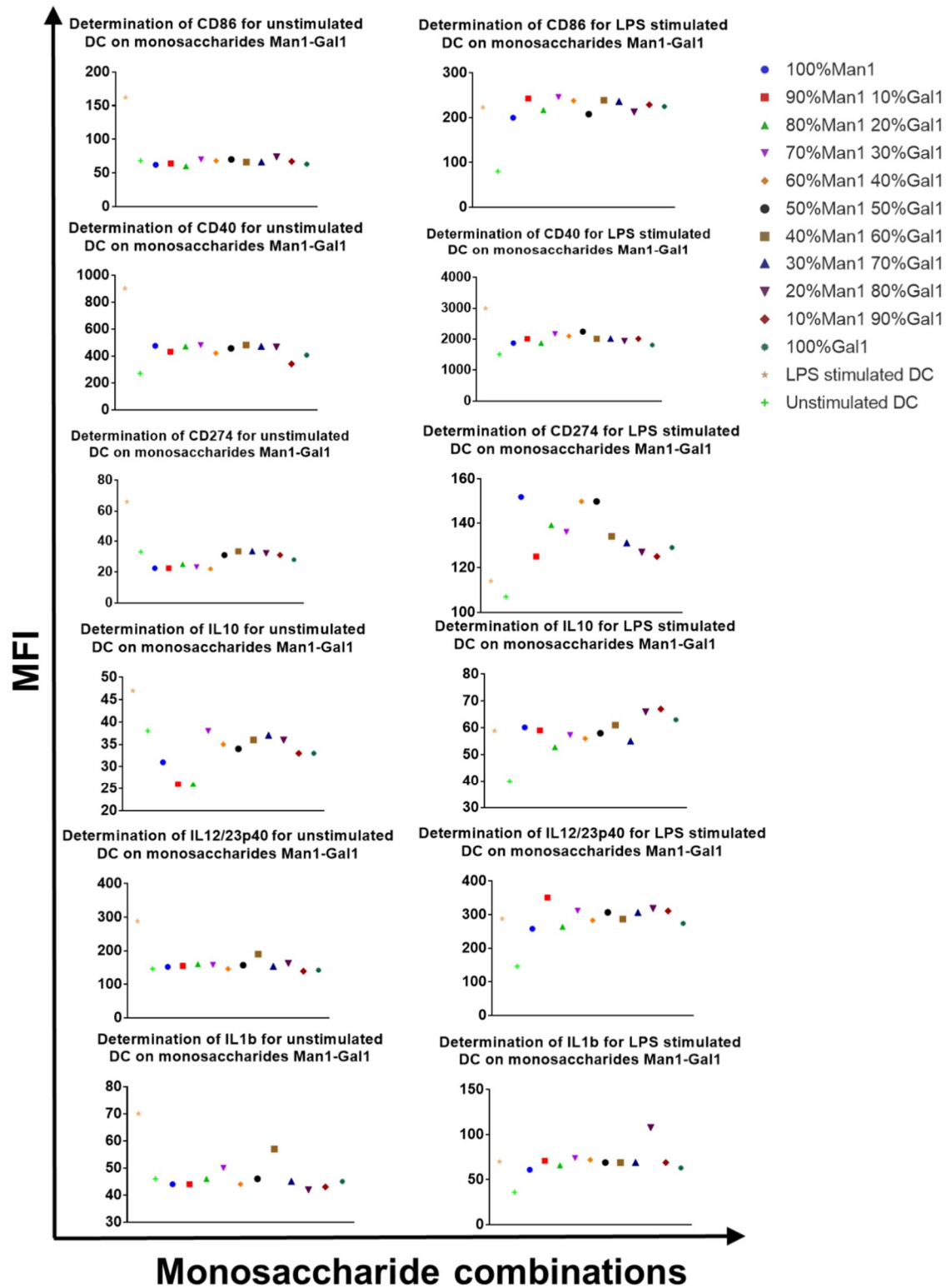
Finally, we have decided to look at conditioned DC-T cell interactions to validate our findings where increased antigen presentation would allow for activated T cell proliferation. Our data show increased number of T cell daughter cells in DC treated with fucose conditions while no increase can be seen in mannose and uncoated AuNPs compared to controls. This suggests increased proliferation for fucose DC-T cells while proliferation remains comparable for mannose and uncoated AuNPs. These findings complement previous our co-localization data and show that rapid lysosomal delivery increase cell presentation which in turn would initiate proliferation of activated T cells. This shows the potential of sAuNPs as promising tools for drug delivery, gene therapy and antigen delivery with enhanced uptake.

In conclusion we were able to prove monosaccharides potential in immunomodulation and drug delivery using both immobilized and polymer attached, respectively. Future work on immobilized monosaccharides could involve autoimmune models to look at HIT primed DCs ability to reduce inflammation. Also understanding the molecular basis of Immobilized monosaccharide HITs ability to generate TH17 and T-reg populations through looking at intracellular pathways including IRF4 would provide more understanding in the field of glycoimmunology. Further experiments on monosaccharide attached AuNPs could be carried to look at delivery of payloads or addition of immunomodulatory molecules in conjugation with monosaccharide for the successful delivery to DCs.

Appendix



Appendix Figure 1: Showing gating strategies for obtaining MFI for each of the channels with appropriate single stain controls (SSC). Once MFI is obtained it is normalized to that of LPS as a percentage for each donor. The data shown is representative 1 of 4 donors.



Appendix Figure 2: Phenotypic analysis of immobilized monosaccharide conditioned iDCs and mDCs. DCs were analysed for maturation and co-stimulation markers (CD86, CD40, inhibitory markers (CD274), pro-inflammatory cytokines (IL-12p40, IL-1 β) and anti-

inflammatory cytokines (IL-10). Data show no significant phenotype or cytokine changes on DCs conditioned with monosaccharides in the absence of LPS. However, anti-inflammatory changes can be noted on LPS stimulated conditioned DCs were cell are skewed to a regulatory phenotype after the treatment with the monosaccharide libraries compared to untreated DCs. This is seen through the increase in IL-10 and CD274 with the decrease in IL-12p40 and CD40 by these cells. LPS induced maturation and stimulation of DCs was not affected which is seen through the unchanged levels of CD86 and sustained production of IL1 β . Data shown are a representative of 2 donors.

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