The role of α2,3- and α2,6-sialylation in functional aspects of dendritic cells

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Tese para obtenção do grau de Doutor em Ciências da Vida,
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“I love it when a plan comes together!” (Adoro quando um plano dá certo!)

- Col. John ‘Hannibal’ Smith, ‘The A-Team’ TV series, as portrayed by actor George Peppard

“Your reason and your passion are the rudder and the sails of your seafaring soul.

If either your sails or your rudder be broken, you can but toss and drift, or else be held at a standstill in mid-seas.

For reason, ruling alone, is a force confining; and passion unattended, is a flame that burns to its own destruction.

Therefore, let your soul exalt your reason to the heights of passion, that it may sing;

And let it direct your passion with reason, so that your passion may live through its own daily resurrection, and like the phoenix rise above its own ashes.

[…]

Among the hills, when you sit in the cool shade of the white poplars, sharing the peace and serenity of distant fields and meadows – then let your heart say in silence, ‘God rests in reason.’”

- in The Prophet, Khalil Gibran

“*Where there’s a will, there’s a way.*” (Querer é Poder.)

- Popular saying and motto of “Instituto Militar dos Pupilos do Exército”

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"We learned about gratitude and humility - that so many people had a hand in our success, from the teachers who inspired us to the janitors who kept our school clean... and we were taught to value everyone's contribution and treat everyone with respect."

- Michelle Obama

"The discipline of gratitude is the explicit effort to acknowledge that all I am and have is given to me as a gift of love, a gift to be celebrated with joy."

- ***Henri Nouwen***

The people who know me personally know that I don't follow the "oft-beaten path" regarding aspects in life that enjoy a certain freedom. The present section is one of them, I believe.

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List of Abbreviations

A

Ab - Antibody

ACN – acetonitrile

ANOVA – Analisys of Variance

APC (cell) – Antigen-presenting cell

APC (fluorochrome) - allophycocyanin

Asn - Asparagine

B

BDCA – Blood Dendritic Cell Antigen

BMDCs – Bone Marrow-derived Dendritic Cells

BSA – Bovine sérum Albumin

CCL – Chemokine (C-C motif) ligand

CCR – Chemokine (C-C motif) receptor

C

CD – cluster of differentiation

Cdc42 – Cell division cycle 42 protein

cDCs – conventional Dendritic Cells

CFSE – carboxyfluorescein succinimidyl ester

CLR – C-type lectin receptor

CMP – cytidine monophosphate

CMP-5-NeuAc – cytidine 5′-monophospho-N-acetylneuraminic acid

CR3 – complement receptor 3

CXCR – Chemokine (C-X-C motif) receptor

D

DAMP – Danger-associated molecular pattern

DCAR – Dendritic Cell immuno-Activating Receptor

DCIR – Dendritic Cell Immunoreceptor

DCs – Dendritic Cells

DC-SIGN – Dendritic Cell-Specific Inter-cellular adhesion molecule-3-Grabbing Non-integrin

DEC-205 – Dendritic and Epithelial Cells, 205kDa (protein)

DNA – Deoxyribonucleic acid

DNGR-1 – Dendritic Cell Natural Killer lectin Group Receptor 1

E

E. coli – *Escherichia coli*

ECM – Extracellular matrix

EDTA – Ethylenediamine tetraacetic acid

ERK – Extracellular signal-regulated Kinase

F

FasL – Fas Ligand

Fc – Fragment crystallizable (of Ig)

FCS/FBS – Fetal Calf/Bovine Serum

FITC – Fluorescein isothiocyanate

FLT-3 – fms-like Tyrosine Kinase 3

FLT-3L – fms-like Tyrosine Kinase 3 Ligand

Fuc – Fucose

G

GADPH – Glyceraldehyde 3-phosphate dehydrogenase

Gal – Galactose

GalNAc – N-acetyl-galactose

GD1a, c – disialic ganglioside

Glc – Glucose

GlcNAc – N-acetyl-glucosamine

GM1 – monosialotetrahexosylganglioside

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GTP – guanosine triphosphate

GTPase – guanosine triphosphatase

H

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

HRP – horseradish peroxidase

I

ICAMs – Intracellular Adhesion Molecules

iDCs – inflammatory Dendritic Cells

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

ITAM – Immunoreceptor Tyrosine-based Activation Motif

ITIM – Immunoreceptor Tyrosine-based Inhibition Motif

J

JAM-3 – Junctional adhesion molecule C

JNK – c-Jun protein kinase

KO – knock-out (genetic)

L

LacNAc – N-acetyl-lactosamine

Le^{y} – Lewis Y antigen

LFA-1 – Lymphocyte function-associated antigen 1

LN – Lymph node

LP – lamina propria

LPS – Lipopolysaccharide

M

MAA – *Maackia amurensis* agglutinin (lectin)

Mac-1 – Macrophage-1 antigen

MAG – Myelin-associated glycoprotein

MALDI-TOF/TOF – Matrix-assisted Laser Desorption/Ionization – Time Of Flight/Time of Flight

MAPK – Mitogen-activated protein kinases

MFI – mean fluorescent intensity

MGL – Macrophage galactose-binding lectin

MHC – Major histocompatibility complex

moDCs – monocyte-derived Dendritic Cells

MR – Mannose Receptor

mRNA – messenger Ribonucleic acid

MS – Mass Spectrometry

MUC1 – Mucin-1

m-moDCs – mature moDCs

N

Neu 1(to 4) – Neuraminidase 1(to 4)

Neu5Ac – N-acetylneuraminic acid

NF-κB – Nuclear Factor kappa-light-chain-enhancer of activated B cells, or, Nuclear factor kappa B

NOD – Nucleotide-binding oligomerization domain (receptors)

O

Ova – Ovalbumin

OX40-L – OX40 Ligand

P

PAMPs – Pathogen-associated molecular patterns

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffer Saline

PBS-T – Phosphate Buffer Saline with Tween-20 added

PCR – Polymerase chain reaction

pDCs – plasmacytoid Dendritic Cells

PE – Phycoerythrin

PerCP – Peridinin-Chlorophyll protein

pERK – Extracellular-signal-regulated kinase, phosphorylated (activated) form

PGE2 – Prostaglandin E2

PHA – Phytohemagglutinin (lectin)

PLT – Primary Lymphoid Tissue

PNA – *Arachis hypogaea* (peanut) agglutinin (lectin)

PRRs – Pattern Recognition Receptors

PSGL-1 – P-selectin glycoprotein ligand-1

PTA – phosphotungstic acid

PVDF – Polyvinylidene difluoride

R

RA – Retinoic Acid

RANTES – regulated on activation, normal T cell expressed and secreted (chemokine)

RNA – Ribonucleic acid

ROR-γt – Retinoic acid-related Orphan Receptor-γ t

STAT3 – Signal transducer and activator of transcription 3

sTn – sialyl-Tn antigen

S

SAMP – Self-associated molecular pattern

SDS-PAGE – Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis

Ser – Serine

Siglec – Sialic acid-binding Immuno-globulin-like lectin

sLe^a – sialyl-Lewis A antigen

sLe^x – sialyl-Lewis X antigen

SLOs – Secondary Lymphoid Organs

SNA – *Sambucus nigra* (elderberry) agglutinin (lectin)

ST – sialyltransferase

ST3Gal-1(to -4) – β-galactoside α2,3-sialyl-transferase 1(to 4)

ST3GalNAc-2(to -6) – α-N-acetylgalactosa-minide α2,6-sialyltransferase 2(to 6)

ST6Gal-1 – β-galactoside α2,6-sialyltrans-ferase 1

ST8Sia – α2,8-sialyltransferase

T

TAC – Tumor-associated antigens

TBS – Tris-buffer Saline

TD – Thoracic duct

TGF – Tumor Growth Factor

Th – T helper (cell)

Thr - Threonine

TLR – Toll-like receptors

TNF – Tumor necrose factor

Treg – regulatory T (cell)

TT – tetanus toxoid

V

VCAMs – Vascular Cell Adhesion Molecules

W

WB – western blot

WT – wild-type (genetic)

Abstract

Glycans decorating cell surface and secreted proteins and lipids occupy the junction where critical host–host and host-pathogen interactions occur. In spite of the wide acceptance that glycans are centrally implicated in immunity, exactly how glycans and their variety and variability contribute to the overall immune response remains poorly defined.

Glycans, frequently terminated by sialic acid residues, may be modified by external factors such as pathogens or upon specific physiological cellular events. The terminal, privileged positions of sialic acid-modified structures makes them key, fundamental determinants for a number of immune receptors with known involvement in cellular adhesiveness and cell trafficking, such as Selectins and Siglecs, with known relevant immune functions. At the time this thesis was initiated, it was established that sialic acids expressed at cell surface could modulate important mechanisms of the adaptive immune responses. Given the key role of dendritic cells (DCs) in the transition from innate to the adaptive immune responses, we anticipated that sialic acids could also modulate important mechanisms of human DCs. DCs have a relevant role in antigen screening and uptake, migration to lymph nodes and antigen presentation to lymphocytes, ultimately triggering the adaptive immune response. Therefore, our primary hypothesis was that sialic acids may modulate DC functions, such as antigen uptake, maturation, homing to lymph nodes and antigen presentation to T cells.

To test this hypothesis, we divided our work in four parts.

1) Surface sialylated glycans expressed during differentiation from human monocytes to DCs (moDCs) were analyzed. Our data showed that α2,3-sialylated O-glycans and α2,6- and α2,3-sialylated N-glycans expression increased during moDC differentiation. Three main sialyltransferases (STs) are committed with this new glycan configuration: ST6Gal-1 correlates with the increased expression of α2,6-sialylated N-glycans; ST3Gal-1

contributes for the α2,3-sialylation of O-glycans, especially T antigens; and ST3Gal-4 may contribute for the increased α2,3-sialylated N-glycans. Upon moDC maturation, ST6Gal-1 and ST3Gal-4 are downregulated and ST3Gal-1 is altered in a stimulus dependent manner.

2) We subsequently analyzed the consequences of the modulation of cell surface sialic acids in DC functions. We observed that removing surface sialic acid by sialidase significantly decreased the capacity of moDCs to micropinocytose and receptor-mediated endocytose. In contrast, treatment with a sialidase significantly improved the capacity of moDCs to phagocytose *Escherichia coli*. The improved phagocytosis mechanism required *E. coli* sialic acids, indicating a mechanism of host–pathogen interaction dependent on sialic acid moieties.

Sialidase-treated moDCs have increased expression of MHC and co-stimulatory molecules, suggesting a more mature phenotype. Experiments using mouse bone marrow-derived DCs (BMDCs) from ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ strains indicated that endocytosis and maturation are influenced by changes in either α2,3 or α2,6-sialylated glycans. The analysis of α2,6-sialylated, N-glycosylated proteins, strongly suggested the potential involvement of β_2 integrins, underlying these mechanisms.

3) The effect of α2,6-sialylation in DC homing to lymph nodes was also analyzed. We observed that BMDCs deficient for ST6Gal-1 have an almost 50% reduction in DC homing, as assayed by *in situ* inflammation and adoptive transfer studies. A reduction in DC homing was also observed when wild type BMDCs were transferred into ST6Gal-1$^{-/-}$ recipient mice. Further investigations are necessary to identify the molecules involved in this process.

4) Finally, we also analyzed the impact of sialylation on DCs ability to prime T cells. Sialidase-treated moDCs show increased gene expression of IL-12, TNF-α, IL-6 and IL-10 cytokines, and activation of the transcription factor nuclear factor-κB. Sialidase-

treated DCs induced a higher proliferative response of T cells with concomitant higher expression of interferon-γ, suggesting that the clearance of cell surface sialic acids contributes to the development of a T helper type 1 proinflammatory response.

Together, our data strongly support sialic acid's relevance in DC immune functions. Alterations of cell surface sialic acid content can alter the endocytosis/phagocytosis, maturation, migration/homing and the ability for T cell priming in human DCs. Moreover, sialic acid linkages created by ST6Gal-1 and ST3Gal-1 are functionally relevant. The engineering of cell surface sialylation, mediated by individual sialidases or sialyltransferases is a likely possibility to fine tune DC phagocytosis and immunological potency, with particular significance to DC-based therapies.

Resumo

Os glicoconjugados que decoram a superfície celular e os lípidos e proteínas secretados ocupam o ponto de encontro onde normalmente ocorrem interacções críticas homólogas (hospedeiro-hospedeiro) e heterólogas (hospedeiro-patogénio). Apesar de ser largamente aceite que os glicanos são parte integrante do processo de imunidade, continua a não ser claro qual o papel que os glicanos, em toda a sua diversidade, tomam no quadro geral da imunidade.

Os glicanos, que são frequentemente terminados por resíduos de ácido siálico, podem ser alterados por factores externos, tais como patogénios, ou por acontecimentos fisiológicos celulares específicos. Normalmente em posição terminal, as glico-estruturas que contêm ácido siálico assumem um papel fundamental numa quantidade substancial de receptores imunes envolvidos na adesividade e tráfico celular, tal como as Selectinas e as Siglecs, das quais se sabe apresentarem uma relevante função imune.

À altura do início desta tese, era sabido que os ácidos siálicos expressos à superfície das células poderiam modular mecanismos importantes nas respostas imunes adaptativas. Considerando a posição de charneira que as células dendríticas (DCs) ocupam na transição da resposta imune inata para a adaptativa, antecipámos que os ácidos siálicos poderiam também modular mecanismos relevantes nas DCs humanas. As DCs têm uma função muito relevante na verificação e captura antigénica, migração para os gânglios linfáticos e apresentação antigénica aos linfócitos, uma sequência de funções que conduz, em ultima instância, à indução da resposta inata adaptativa. Considerando estas premissas, a nossa hipótese principal foi que os ácidos siálicos podem influenciar funções relevantes das DCs, tais como captura de antigénios, maturação, migração para os gânglios linfáticos e apresentação antigénica às células T.

Para testar esta hipótese, dividimos o trabalho em quatro partes:

1) Analisámos os glicanos sialilados de superfície, expressos durante a diferenciação de monócitos humanos em DCs (moDCs). Os nossos dados mostraram que a expressão dos glicanos com ligações em O (O-glicanos) e sialilados em α2,3, assim como glicanos com ligações em N (N-glicanos) sialilados em α2,6 e α2,3 aumentou durante o processo de diferenciação das moDCs. Contribuindo para esta nova configuração glicosídica, três sialiltransferases (STs) poderão estar envolvidas: a ST6Gal-1 correlaciona-se com a expressão aumentada de N-glicanos sialilados em α2,6; a ST3Gal-1 contribui para a sialilação em α2,3 de O-glicanos, em especial de antigénios T; e a ST3Gal-4 poderá ser responsável pelo aumento de N-glicanos sialilados em α2,3. Após estímulo e consequente maturação das moDCs, ambos os níveis de expressão génica de ST6Gal-1 e ST3Gal-4 são negativamente modificados sendo, também, que a expressão de ST3Gal-1 varia consoante o estímulo.

2) Estudámos posteriormente as consequências da modulação dos ácidos siálicos de superfície nas funções das DCs. Observámos que a remoção dos ácidos siálicos de superfície diminui significativamente a capacidade de macropinocitose e endocitose mediada por receptores nas moDCs. Em contrapartida, o tratamento com sialidase aumentou significativamente a capacidade das moDCs para fagocitar *Escherichia coli*. Determinou-se também que este mecanismo requer a existência de ácido siálico presente nas *E. coli* indicando um mecanismo de interacção hospedeiro-patogénio dependente de ácido siálico em ambas as partes envolvidas.

As moDCs tratadas com sialidase também apresentam um nível superior de expressão de moléculas de MHC e moléculas co-estimulatórias, sugerindo um fenótipo celular mais maduro. Recorrendo ao modelo de ratinho, utilizaram-se DCs derivadas de células da medula (BMDCs) de ratinhos deficientes em ST3Gal-1 e ST6Gal-1. Estes ensaios revelaram que quer a endocitose quer a maturação são influenciadas por modificações

nos glicanos sialilados em α2,3 ou α2,6. A detecção e quantificação de proteínas N-glicosiladas e sialiladas em α2,6 apontou para um potencial envolvimento de integrinas β_2 nestes mecanismos.

3) O efeito da sialilação em α2,6 na migração das DCs para os gânglios linfáticos foi também analisado. Observámos que BMDCs deficientes para ST6Gal-1 apresentam uma redução de cerca de 50% nos níveis de migração das DCs para os gânglios linfáticos, tal como aferido em ensaios de inflamação *in situ* e estudos de transferência adoptiva de células. Uma redução dos níveis deste tipo de migração foi também observada quando BMDCs nativas foram transferidas para ratinhos receptores deficientes em ST6Gal-1. São, contudo, necessários mais ensaios de forma a identificar as moléculas envolvidas neste processo.

4) Por último, analisámos o impacto da sialilação na estimulação antigénica das DCs às células T. Assim, concluiu-se que moDCs tratadas com sialidase apresentam um nível de expressão superior de IL-12, TNF-α, IL-6 e IL-10, e activação do factor de transcrição nuclear kappa B (NF-κB). As DCs tratadas com sialidase induziram uma maior proliferação nas células T, com expressão correspondente de interferão-γ. Este dado sugere que a remoção de ácidos siálicos de superfície contribui para o desenvolvimento de uma resposta pro-inflamatória do tipo 1 por células T auxiliares (resposta T_h1).

Considerando estes dados no seu todo, concluímos que o ácido siálico tem um papel marcante nas funções imunes das DCs. Alterações à concentração de ácido siálico à superfície das células podem alterar a endocitose/fagocitose, maturação, migração para os tecidos e gânglios linfáticos e capacidade estimulatória para com as células T. Complementando estes dados, as ligações glicosídicas de ácidos siálicos criados por ST6Gal-1 e ST3Gal-1 são funcionalmente relevantes. A modulação programada da sialilação do glicocálice, mediada por sialidases individuais ou sialiltransferases é uma

possibilidade aceitável para a melhoria da fagocitose por DCs e da sua potência imunológica. Este facto tem um significado particular para imunoterapias baseadas em DCs, podendo provar-se decisivo para a sua eficiência e aplicabilidade num futuro muito próximo.

Section 1

INTRODUCTION

Dendritic cells (DCs) are key players of the immune system. As antigen-presenting cells, they occupy the intersection between innate and adaptive immunity. By surveying the body microenvironment, they are strategically positioned to capture antigens and correctly classify collected antigen information, in a 'self' or 'foreign' category, and to respond accordingly. They carry antigen information from the infection site to the secondary lymphatic organs, presenting them to T cells, thus promoting an adaptive immune response against a specific antigen. The immune response against pathogens greatly depends on the function of DCs. Moreover, DCs also play an important role in anti-tumor immunity, in which specific cytotoxic T cells are primed by DCs to respond against tumor cells.

In accordance with the wide variety of immune scenarios that DCs abridge, different DC subsets have been described. Such classification is made based in their origin, phenotypic profile, and physiological properties.

Impaired DC functions may lead to several pathologies, such as increased susceptibility to infection and cancer or, oppositely, to a wide range of autoimmune diseases (Collin et al., 2011). Investigating the underlying mechanisms of DC-pathogen or DC-host and -tumor cell interactions may help us to better comprehend the immune response in physiological and pathological events and to identify new targets for therapeutic intervention.

Glycosylation is the most frequent post-translational modification of all secreted and cell surface proteins, as well as of lipids. Specific glycan patterns, or signatures, are present on the surfaces of DCs. These signatures are altered not only during differentiation but also in response to cytokine signals and antigen stimuli (Bax et al., 2007). **Sialic acid is a sugar moiety that frequently terminates glycan structures**. Due to its terminal position sialic acid is recognized or modulates ligand recognition by several immune receptors. Therefore sialic acid might modulate many immune processes such as host-pathogen recognition, cell migration, and antigen-

presentation (Varki, 1997;2008). The addition of this sugar is mediated by the **sialyltransferases**, enzymes that are mainly located in the Golgi apparatus. Sialyltransferase expression is finely regulated during DC differentiation and maturation, concurring with the expression of specific sialylated structures.

In diverse immune events, the sialylated glycans will be recognized by receptors, i.e. **lectins**, such as **Siglecs**. While promoting cell recognition by some lectins, the presence of specific sialic acids can also switch off recognition by other lectins specific for asialylated glycans, such as **galectins**. Thus, recognition of sialylated glycans may impact the DC immunobiological functions. The clarification of sialic acid's influence in the DC immunobiology, thus, will potentially lead to a better understanding of the immune mechanisms mediated by DCs and may reveal novel therapeutic means to modulate immune responses.

Therefore, **the scope of this thesis is to study how glycans modulate DC-mediated functions, with special attention to the sialic acid-mediated ones and how they modulate the different DC functions**. This chapter includes an introduction of DCs' function and glycan-recognition receptors, characterization of DCs' sialome-related factors, followed by a description of processes known to be modulated by sialic acid such as endocytosis, migration, priming of adaptive immune response and a very short approach on pathogen/tumoral recognition.

1.1 Immune system overview

Living beings, evolutionarily, developed strategies to shield themselves against the negative effects of pathogens. One such strategy is the immune system, comprising specific sets of cells and molecules dedicated to protect our body against the harm caused by pathogens and toxins. Together, these specific immune components orchestrate a conceptual state commonly termed immunity.

Immunity, as a concept, can be traced as far as the times of the first great Asian and Mediterranean empires upon the simple observation that following contraction of a certain diseases (such as smallpox or some variants of plague), its survivors would no longer be affected by that illness, even when in direct contact with other affected individuals. However, immunology as a science would only begin to develop from the industrial Revolution onwards with small but decisive steps taken by doctors and scientist such as Robert Koch, Louis Pasteur or Edward Jenner. Also decisive were the works of Elie Metchnikoff, Erin von Behring and Shibasaburo Kitasato, in the 19th century, whose observations led to the conclusion that the immune system comprises two different types of response: non pathogen-specific agents like macrophages (one of the first uncovered immune cells), existing prior to any infection, and specific pathogen recognizing molecules such as antibodies, present only after contact with a pathogen. These observations are at the root of the today widely accepted concept of innate and adaptive immune responses. Yet, one would need to wait for the 20th century to unveil the majority of the cellular participants of this immune play and its molecular components, and to establish one of main principles of the immune system: the relation between the innate and adaptive immune response.

1.1.1 The innate immune response

The first line of immune system defense against biological aggression is the innate immune response. The main function of this phase is to provide an immediate response in order to contain the infection and to avoid the development of the pathology. During the innate immune response, antigenic information is collected and presented to lymphocytes, in order to stimulate a specific, enduring adaptive response (Janeway, 1989).

The innate phase response is characterized by a set of cellular and biochemical defense mechanisms, namely, 1) physical and chemical barriers, i.e. epithelial tissues and its secreted anti-pathogenic substances; 2) cells, i.e. phagocytes, granulocytes, and NK cells; 3) serum proteins, i.e. complement proteins, innate antibodies and inflammatory mediators; and 4) the coordinating/regulating, leukocyte-messaging molecules, i.e. cytokines and chemokines (Abbas et al., 2010).

1.1.1.1 Bridging the adaptive immune response

Another important function of the innate immune response is to elicit the longer duration and more effective adaptive immune response. This function is mediated by phagocytic cells such as the Dendritic Cells (DCs) that capture the pathogens process them, and present them to T cells in secondary lymphoid organs. The co-localization of phagocytes and T cells in secondary lymphoid organs after antigen challenging was first shown by Sharp et al. (Sharp and Burwell, 1960), followed by evidence of clear interaction between these cell types (Schoenberg et al., 1964). Later, it became clear that the activation of T cells requires histocompatibility between the antigen-presenter and the T cell (Rosenthal and Shevach, 1973). Although the Major Histocompatibility

Complex (MHC) loci were uncovered in 1951 (Snell and Higgins, 1951), the premise that MHC-restricted antigen presentation was necessary for T cell activation was only confirmed in 1977 (Thomas and Shevach, 1977), thus emerging the concept of antigen presentation, currently accepted. Completing this concept, later observations concluded that different antigen presenting cells (APCs) express different levels of antigen presentation molecules (De Bruijn et al., 1992;Vidard et al., 1992;Robadey et al., 1996) and consequently, different stimulation abilities. Among APCs, it soon became clear that **DCs are the most potent inducers of T cell activation** (Steinman and Witmer, 1978;Van Voorhis et al., 1982;Setum et al., 1993;Mellman et al., 1998).

1.1.1.2 Dendritic cells

DCs are part of the innate immune response and are essential to boost and/or regulate the adaptive immune response - therefore, **DCs sit at the crossroads of both innate and adaptive immune responses**.

DCs capture antigens in an earlier phase, process them 'on the go' while migrating towards secondary lymphoid organs, such as lymph nodes, where they present the processed antigens to T cells via major histocompatibility complex (MHC), the processed antigens to T cells thus enacting an adaptive immune response (Steinman and Cohn, 1973;1974;Steinman et al., 1974;Steinman et al., 1975;Steinman and Witmer, 1978;Steinman et al., 1979). DCs can also present antigens to B cells, although by non-classical (non-MHC) mechanisms (Palucka and Banchereau, 2002;Qi et al., 2006;Harwood and Batista, 2010) (Fig. 1.1).

Morphologically, DCs are relatively large cells with varying numbers of heterogeneously shaped pseudopods. Given these variety of shapes these cells were coined "dendritic" cells (Steinman and Cohn, 1973).

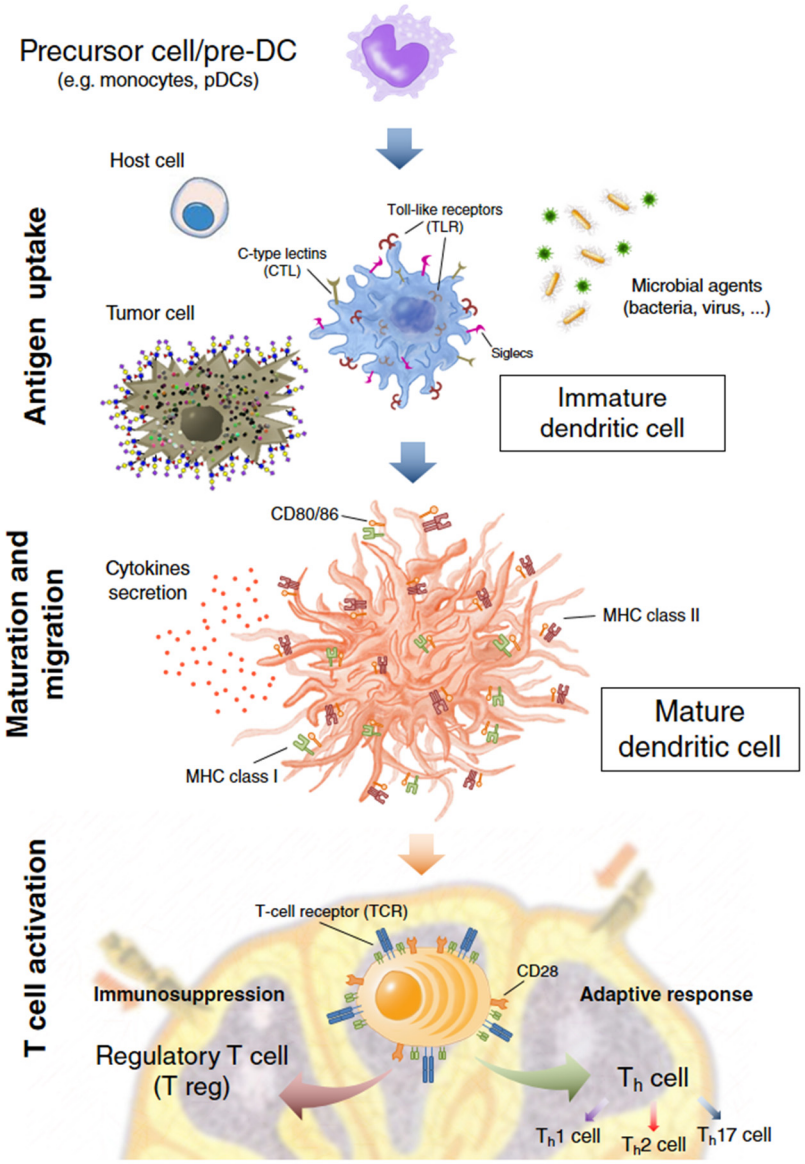


Fig. 1.1 – Basic hallmarks and functions of dendritic cells (DCs). *In Crespo HJ et al.* "Dendritic cells: a spot on sialic acid", *Frontiers in Immunology (4), 2013.*

1.1.1.2.1 DC subtypes and their hematopoietic differentiation

Different DC subsets are widespread throughout the organism, inhabiting different organs and tissues such as the liver, spleen, thymus, gastrointestinal tract or skin, and many more, phenotypically adapted to the tissue they reside. **DC cell surface present higher expression levels of MHC class II and co-stimulatory molecules, such as CD80 and CD86 (B7.1 and B7.2) and CD40,** than other immune cells, such as

macrophages or B cells. This fact is highly relevant for their antigen-presenting function (van der Valk et al., 1984;Wood et al., 1985;Landry et al., 1988).

DCs can also be phenotypically characterized by the expression of specific markers: **B220, CD103 and CD8** in mouse, and the **D**endritic **C**ell-**S**pecific **I**ntracellular adhesion molecule 3 (ICAM-3)-**G**rabbing **N**onintegrin (**DC-SIGN**), **CD123**, **CD1c/Blood Dendritic Cell Antigen-1** (**BDCA-1**), **CD141/BDCA-2** and **BDCA-3** and **-4** in human (Dzionek et al., 2000;MacDonald et al., 2002;Wollenberg et al., 2002;Robbins et al., 2008;Haniffa et al., 2009;Bachem et al., 2010;Crozat et al., 2010;Jongbloed et al., 2010;Poulin et al., 2010;Reizis et al., 2011;Haniffa et al., 2012;Langlet et al., 2012;Satpathy et al., 2012;Lundberg et al., 2013;Plantinga et al., 2013). **CD11b and CD11c** are also DC markers widely used on mouse DC phenotyping that are also human DC markers, but not exclusively.

In the absence of infection and inflammation, DCs are functionally divided in two major types: **migratory** and **non-migratory, or lymphoid, tissue-resident** DCs (reviewed in (Shortman and Naik, 2007;Collin et al., 2013)). A classic example of the former are **dermal DCs and Langerhans cells** that mainly reside in skin tissues. Upon contact with antigen, they mature and migrate to the draining lymph nodes (Silberberg et al., 1974;Ross et al., 1994;Ebner et al., 1998). The **non-migratory DCs** (like spleen or thymus DCs) **reside in secondary lymphoid organs**, where they constantly screen blood or lymph for pathogens (Ardavin, 1997;Henri et al., 2001). Both the canonical myeloid and lymphoid hematopoietic progenitors contribute to the steady-state DC pool. As a common trait, the expression of the **Fms-like tyrosine kinase 3 (Flt-3) molecules is characteristic of DC precursors, regardless of the myeloid or lymphoid lineage and DCs development is driven by Flt3-ligand (Flt-3L)** (Manz et al., 2001;D'Amico and Wu, 2003;Shigematsu et al., 2004;Shortman and Naik, 2007;Watowich and Liu, 2010;Singh et al., 2012). Much interestingly, it was recently reported that steady-state

conventional DCs are marked by the exclusive expression of the DNGR-1 (Schraml et al., 2013) which could, thus, function as a true lineage DC marker.

Opposed to the steady-state conventional DCs, some DCs are classified as **inflammatory or infection-derived DCs**. These populations include the **plasmacytoid DC (pDC)** population, a first line of defense against microbial invasion. Functionally specialized in the **detection of viral infections**, pDCs develop a fully differentiated DC phenotype after infection and secrete type I interferon (Grouard et al., 1997;O'Keeffe et al., 2002). Other inflammatory DCs of interest are the **monocyte-derived DCs** (**moDCs**) (Segura and Amigorena, 2013)). MoDCs are one of the most relevant, well-established **human DC model,** especially in inflammation/pathogenic studies (Romani et al., 1996). This comes as the result of two factors: its relative ease to obtain from monocytes present in peripheral blood (Chapuis et al., 1997;Caux and Dubois, 2001) and the presence of all canonical DC phenotypical and functional properties (maturation markers, endocytosis, allogeneic and/or syngeneic T-cell stimulation) (Akagawa, 1994;Romani et al., 1996;Palucka et al., 1998;Santin et al., 1999).

In mouse, the main DC model is the **Bone Marrow-derived Dendritic Cells (BMDCs)**, allowing the reproduction of the differentiation of different DC subsets, by using different cytokine cocktails (Inaba et al., 1992;Shurin et al., 1997;Zhang et al., 1997;Yamaguchi, 1998;Lutz et al., 1999;Zhang et al., 1999;Brasel et al., 2000).

In the work here presented, these will also be the two DC models of choice as they are the best models that generally represent the DC populations and common functions of all DC subtypes above referred.

1.1.1.2.2 Endocytosis

A fundamental function of DCs is to capture pathogens and then trigger the adaptive response against them, as previously stated. Thus DCs serve a critical frontline function in immunity: the continuous survey of the surroundings by constitutive uptake of

encountered antigens (Sallusto et al., 1995). The three principal means by which DCs capture antigens are: 1) **receptor-mediated endocytosis**, in which particles are endocytosed after a specific cell-surface receptor recognition; 2) **macropinocytosis**, or the non-selective endocytosis of solutes, a constitutive process and the major source of antigens for presentation by DCs (Norbury, 2006); and 3) **phagocytosis**, the uptake of large molecules or cells, including virus, bacteria, protein clusters, apoptotic and necrotic cells, which also involves specific membrane receptors (Fig. 1.2).

The uptake of foreign antigens usually triggers activation signals that will lead DCs into a mature phenotype that maximizes the potential for antigen presentation and stimulation of the adaptive response immune cells.

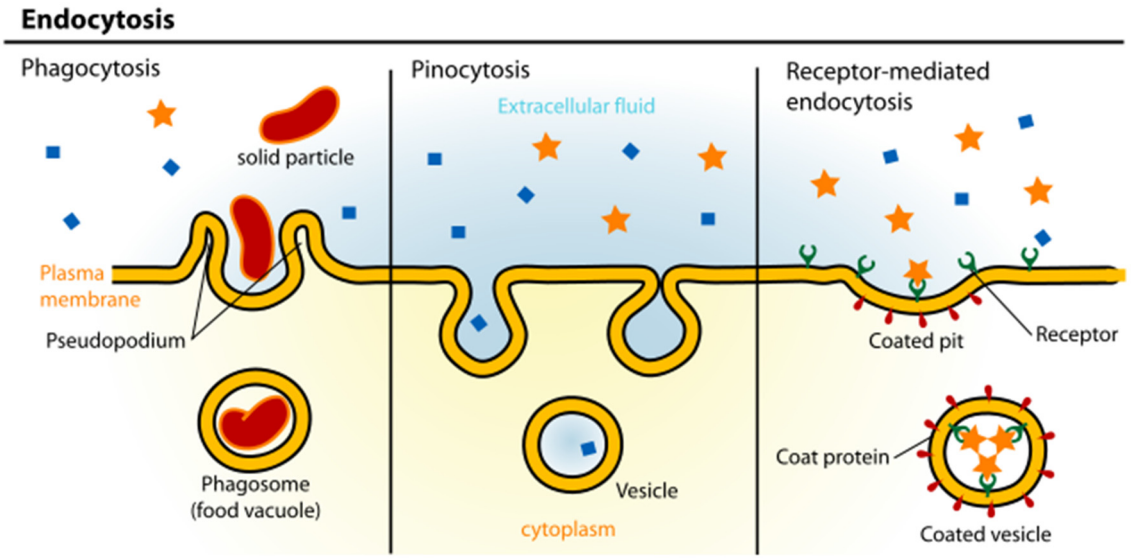


Fig. 1.2 – Types of endocytosis.

Receptor-mediated endocytosis is also fundamental in the maintenance of the self-tolerance mechanisms. At steady-state, self-antigens are endocytosed and posteriorly presented by DCs, but the **endocytosis of self-antigens does not usually induce significant maturation changes** (Wilson et al., 2003).

Defective maturation contributes to turn DCs tolerogenic and promoting regulatory T cell responses. Nevertheless, very small foreign and more common antigens, when present at time-persistent concentrations can also induce tolerance.

1.1.1.2.3 Maturation

DC maturation is the sum of all the phenotypical and functional changes occurring upon encounter with immune stimuli (i.e., antigens, cytokines, etc.) and it is crucial to enable DCs to effectively activate T cells. It is characterized by **rapid downregulation of the antigen-uptake process, acidification of lysossomal compartments, higher expression of MHC II molecules and of CD80 and CD86 co-stimulatory molecules, *de novo* or up-regulated synthesis of DC-specific inflammatory cytokines** (Thomas and Lipsky, 1994). All these maturation and migration-changes are necessary hallmarks to enable DCs to perform antigen presentation and boosting T and B cell responses (Sallusto and Lanzavecchia, 1994). It is also known that the molecular nature of uptaken antigens, as well as the cytokines to which DCs are exposed during the uptake process, are responsible for the modulation of the maturation process. This ultimately influences the differentiation of the DC-pulsed T cells into functionally distinct subtypes, actively shaping a future active or tolerance response.

1.1.1.2.4 Migration

DC migration comprises the whole of the trafficking of DCs: their hematopoiesis on bone marrow, their entry in circulation via blood towards the peripheral tissues and, from here or directly from blood, to the secondary lymphoid organs (Fig. 1.3).

The homing of conventional or inflammatory DCs loaded with antigens to T cell niches (normally, secondary lymphatic organs) is a crucial step for the setting of effective immune responses. This process is characterized by **chemokine-mediated cell-recruitment to the lymphoid target site and activation of the surrounding tissues** (von Andrian and Mempel, 2003;Bonasio and von Andrian, 2006;Forster et al., 2012). Tissue activation helps to increase the cell adhesion to the endothelium, by inducing the

expression of several adhesion molecules, of which integrins and selectins and its ligands are the most relevant elements.

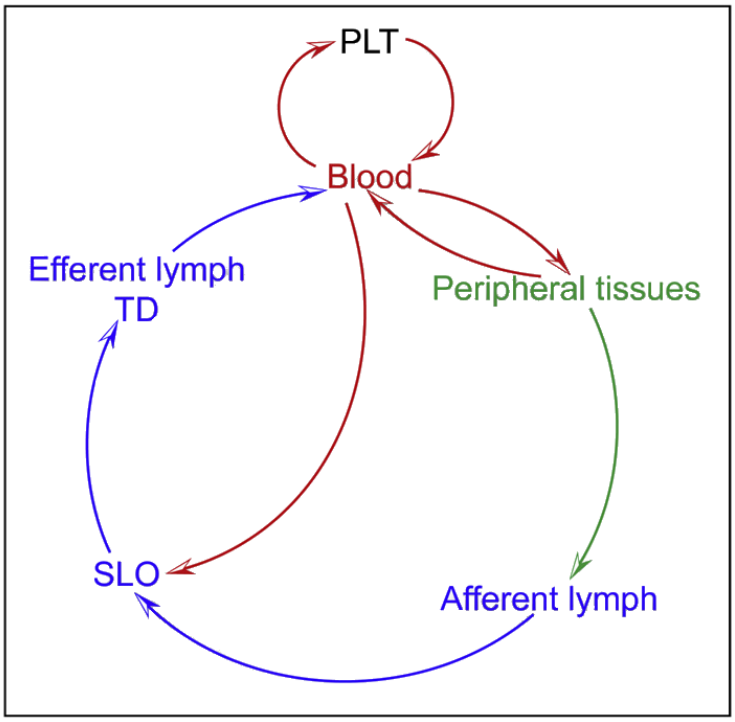


Fig. 1.3 - Programmatic Outline of DC and DC-Precursor Trafficking Routes. DCs develop from precursors that originate from primary lymphoid tissues (PLT) such as the BM and the thymus. Precursors and committed DCs enter the circulation and seed peripheral tissues and secondary lymphoid organs (SLOs). From peripheral tissues, they can access afferent lymph upon receiving a mobilization signal and travel to the draining LN. Leukocytes leave LNs via the efferent lymph and are collected in the thoracic duct (TD), which eventually guides DCs and their precursors back into the circulation. *In Alvarez D. et al*., Mechanisms and Consequences of Dendritic Cell Migration, *Immunity, 29, 2008*.

DCs also migrate to non-lymphoid tissues, although this form of migration process and causes are, still, not totally clear. A steady-state setting is an unfavorable environment for DCs or DC precursors to migrate to non-lymphoid peripheral tissues. Although the rolling and tethering of DCs are observable in both steady-state and inflammatory contexts, migration to non-lymphoid tissues requires many of the adhesion molecules (intracellular adhesion and vascular adhesion molecules (ICAMs and VCAMs), integrins) normally involved in migration and homing phases that are ordinarily induced by inflammatory mediators (Robert et al., 1999b;Merad et al., 2002;Pendl et al., 2002;Merad et al., 2004). Elucidating the underlying mechanisms of DCs and/or their

precursors' migration in the steady state (or inflammation-suppressed pathologies, like several neoplasias) could prove to be of extreme importance in DC-based therapies, where this DC attribute needs further improvement.

1.1.1.2.5 Cytokine production

DCs provide the lymph node-based naive T helper (T_h) cells with two pathogen-related signals: the information about the structure of pathogen's antigens and its pathogenicity. This comes as the result of the endocytosis and subsequent antigen-processing during homing towards the lymph nodes. Upon arrival in the lymph nodes, **mature DCs can then present the tissue-derived pathogen information to provide T cells via three signals**: an Ag-specific '**signal 1**' – MHC:T cell receptor triggering; a '**signal 2**', resulting from co-stimulatory stimulus, transmitting information regarding pathogenic potential; and a '**signal 3**', comprising cytokine production (Howard et al., 2002;Corthay, 2006). The latter provides a first, determinant impulse towards one of the T_h profiles, namely, **T helper type 1 or 2 (T_h1 or T_h2)** – responsible for the stimulation of a specific cellular and humoral response (respectively) –, **T helper 17 (T_h17)** – stimulating anti-microbial mechanisms on epithelial and mucosal barrier tissues –, **or regulatory (T_{reg}) cells** – inducing, as the name suggests, immune tolerance towards elicited antigens (Cools et al., 2007).

In very general terms, **IL-12 and IL-4 cytokines are the most relevant cytokines involved in T cell priming**, with IL-12-producing DCs priming T_h1 responses, and the non-IL-12-producing DCs priming T_h2 responses (Maldonado-Lopez et al., 1999). The main drivers onto T_h1 are **inflammatory, monocyte-derived CD11c$^+$ DCs** (Macatonia et al., 1995;Rissoan et al., 1999), and **CD11c$^-$ cDCs** into T_h2 (Rissoan et al., 1999).

The T_h1-skewing cytokine IL-12 is the best explored, most relevant pro-inflammatory third signal in DCs (Trinchieri, 1995), with IFN-γ, IL-4, IL-10, PGE2 and IFN-α activities intimately mediated by this cytokine (Gately et al., 1998;O'Garra, 1998;Wu et al., 1998). Its main inducers are lipopolysaccharide (LPS), artificially synthesized poly(I)poly(C) or CD40L (Macatonia et al., 1995;Cella et al., 1996;Cella et al., 1999).

Modulation of IL-12 expression also can be achieved by the action of chemokines, either positively, such as in the lymphoid-resident DCs in the mouse model (Aliberti et al., 2000), or negatively, as in the case of human DCs (Braun et al., 2000). DC contact with most pathogens induces IL-12 production. In contrast, maturation stimuli such as TNF-α, IL-1, cholera toxin, FasL, fungal hyphae or nematode products seem to have no impact in this cytokine secretion (Braun et al., 1999;d'Ostiani et al., 2000;Gagliardi et al., 2000;Rescigno et al., 2000;Whelan et al., 2000). IL-12 production can be potently induced by CD40L, which is expressed at high levels on activated memory T cells (Cella et al., 1996;Heufler et al., 1996). In terms of activation kinetics, DCs only respond within 8 to 16 hours after challenge with IL-12-inducing stimuli. This activation lag ensures a regulation of T_h1 and T_h2 polarization, avoiding unwanted responses (Langenkamp et al., 2000;Lanzavecchia and Sallusto, 2000;Tanaka et al., 2000).

The **T_h2-skewing in DCs profile is characterized** by secretion of **low levels of IL-1α/β, IL-6, IL-13 or OX40L**, but, also importantly, by the **absence of IL-12** (Rincon et al., 1997;Flynn et al., 1998;McKenzie et al., 1998;Rissoan et al., 1999), resulting from **DC contact with low stimulatory antigens**. Recently, it was shown a normally tolerated antigen became susceptible of a T_h2 response after infection with a strong T_h1-elliciting pathogen (Brimnes et al., 2003;Dahl et al., 2004), a fact that further stresses how complex and unclear the T_h2 process still is.

DCs are also known to actively participate in the induction of T_{reg} cells (Fairchild and Waldmann, 2000;Steinman et al., 2000). The induction of tolerance, more than the result of a dedicated, tolerogenic DC lineage or immature DCs, occurs after DC contact with micro-environmental factors (mostly antigens), inducing DCs to a tolerogenic-skewing profile. Good examples of these tolerogenic factors, corroborating this hypothesis, are:

- **Non-immunogenic antigens** present in **respiratory and digestive tracts** (Vermaelen et al., 2001) **Antigens from specific pathogens** - i.e. ***Plasmodium* spp. antigens;**
- **Some self-antigens,** such as **insulin** (Chen et al., 2003);
- **Altered "self" peptides (Wildbaum et al., 2002) or self-antigens resulting from regular cell turnover** (Huang et al., 2000).

Tolerance induction may also originate in pro-inflammatory DCs, as well as specialized subsets/tissue-resident of DCs. For example:

- Activation of DCs that secrete IL-10 (an immunoregulatory cytokine), but not IL-12, can direct naïve T cells to a T_{reg} subtype (Akbari et al., 2001;McGuirk et al., 2002), as in the case of APCs from the liver (Khanna et al., 2000) and Peyer's patches (Iwasaki and Kelsall, 1999) secreting high levels of IL-10, selectively inducing T_{reg} or T_h2 cells, respectively;
- *In vitro* culture of mouse bone marrow progenitor cells **in the presence of GM-CSF, TNF-α and IL-10** induces the differentiation of a distinct subset of dendritic cells, **$CD11c^{low}$ $CD45RB^{+}$ DCs**, similar to a tolerogenic subset naturally/physiologically present in the spleen and lymph nodes of normal mice (Wakkach et al., 2003). These DCs were shown to secrete **high levels of IL-10** after activation. Functional studies also showed that $CD11c^{low}$ $CD45RB^{+}$ DCs specifically induce the differentiation of regulatory T_{reg} cells *in vitro*.

- The **murine CD103$^+$ cDCs**, present in hepatic, skin, kidney, intestinal and lung tissue (Johansson-Lindbom et al., 2005;del Rio et al., 2007;Jaensson et al., 2008;Monteleone et al., 2008;Ginhoux et al., 2009;Schulz et al., 2009;Desch et al., 2011;Scott et al., 2011;Murakami et al., 2013;Yu et al., 2013) were proven to be **relevant T_{reg} inducers via a TGF-β and retinoic acid (RA) mechanism** (Coombes et al., 2007). This mouse subset has recently been shown to be **the functional homologue of the human CD141$^+$ DCs** (Jongbloed et al., 2010;Haniffa et al., 2012;Kelly et al., 2013), with similar T_{reg} induction mechanisms, namely and mainly, via TGF-β with RA also playing a part (Mucida et al., 2007; Nolting et al., 2009).

As an exogenous factor, **IL-10 is, thus, the key factor for the induction of tolerogenic DC *in vitro* and *in vivo***, but other cytokines and factors produced in the peripheral tissues, such as PGE2, TGF-β, RA or endocrine factors should also be taken considered on the generation of tolerogenic DCs (Wilbanks et al., 1992;Hosoi et al., 1993).

More recently, **a newly defined lineage of T cells called T helper 17 (T_h17) cells**, was identified with **protection functions against some bacterial and fungal infections** (Korn et al., 2009) and with a relevant role in autoimmune disorders, such as multiple sclerosis (Correale and Farez, 2012).

T_h17 cells may be induced by DCs as the result of a combination of IL-6, low concentration of TGF-β, IL-23, or IL-21 (Acosta-Rodriguez et al., 2007;Korn et al., 2007;Nurieva et al., 2007;Zhou et al., 2007;Volpe et al., 2008;Yang et al., 2008a;Hu et al., 2011), **with IL-1 also known to enhance this process** mediated by the transcription factor RORγt and the signal transducer and activator of transcription 3 (STAT3) (Bettelli et al., 2006;Mangan et al., 2006;Veldhoen et al., 2006;Manel et al., 2008;Yang et al., 2008b;Ivanov et al., 2009).

However, IL-6 action may be a lineage dependent factor for T_h17 induction. Although both splenic DCs and intestinal lamina propria DCs induce differentiation of naive T cells into T_h17 cells, **splenic DCs do so independent of IL-6**, whereas in the presence of LP and skin $CD103^+$ DCs, **IL-6 is required** to induce T_h17 lineage cells. This comes as a result of the necessity of IL-6 to counteract the anti-T_h17 differentiation effect of RA and **high concentrations** of TGF-β, both known to be produced by skin and intestinal lamina propria (LP) $CD103^+$ DCs (Coombes et al., 2007;Mucida et al., 2007;Manel et al., 2008;Nolting et al., 2009) but not by spleen DCs (Hu et al., 2011).

1.1.2 Pathogen recognition by dendritic cells

Pathogen recognition by DCs depends on the identification of distinct microbial patterns, not present in mammalian cells, but shared by most of the pathogenic microbial, known as '**pathogen-associated molecular patterns**' (**PAMPs**) (Schnare et al., 2000;Netea et al., 2004). These patterns include **bacterial and viral unmethylated CpG DNA**, **bacterial flagellin**, **peptides containing N-formylmethionine residues**, **lipoteichoic acids** and **double-stranded and single-stranded viral RNA**. A substantial part of PAMPs are **glycan-containing** ones, such as **lipopolysaccharide, N-acetylglucosamine, peptidoglycan, terminal fructose- and mannose-containing glycans**, and **glucan-containing cell walls from fungi**.

PAMPs are recognized by specific receptors named '**pattern recognition receptors**' (**PRRs**), with functions aggregating endocytosis and intracellular signaling. Examples of PRRs expressed by DCs include **Scavenger receptors**, **NOD-like receptors** and **C-type lectins**. However, among the most widely studied are the **Toll-like receptors (TLR)**, a growing family of 12 evolutionary conserved PRRs consisting of type I integral membrane glycoprotein with relevant role in the microbial response. The outcome of TLR recognition is the induction of intracellular signaling and consequent expression of

antigen presentation molecules (MHC class II molecules), co-stimulatory molecules (CD80/86, CD40), inflammatory and/or antiviral cytokines (such as TNF-α, IL-12, IL-23, IFNα/β) and chemokines (i.e., IL-8, RANTES) (Takeda et al., 2003;Dzopalic et al., 2012), thus enacting a powerful response against pathogenic microbes.

C-type lectins (CLRs) are another very relevant family of PRRs expressed by DCs (Zelensky and Gready, 2005). As lectins, their main function is **the recognition of glycan structures** and, in an immunological context, they recognize pathogen-associated glycans or glycosylated self-antigens. In DCs, some CLRs of note include the **DC-Specific Intracellular adhesion molecule-3 Grabbing Non-integrin (DC-SIGN)**, **CD207/Langerin**, the **Selectin family** (discussed below), the **Macrophage Galactose/N-acetyl-galactosamine-specific Lectin (MGL1)**, **Mannose Receptor (MR)**, **DEC205**, the **Blood DC antigens 2 (BDCA 2)**, the **Dendritic Cell Immunoreceptor (DCIR)**, **the Dendritic Cell Immunoactivating receptor (DCAR)** and **Dectin-1/2/3**. In contrast to TLRs, all of these CLRs functionally bind glycan structures expressed by mammalian cells (except for Dectin-1/2/3 that apparently only recognizes fungal and/or mycobacterial glycans), a fact that demonstrates its potential role in both host and pathogen recognition (van Kooyk and Rabinovich, 2008). CLRs can also recognize and internalize pathogens for presentation without inducing DCs' maturation. In fact, the CLR-mediated antigen uptake doesn't necessarily elicit a factual immune response, and may instead contribute to induce immunological tolerance (Figdor et al., 2004). A downside of these phenomena is the potential immune escape of pathogens recognized via CLRs (van Kooyk et al., 2004;van Gisbergen et al., 2005;van Kooyk, 2008;van Kooyk and Rabinovich, 2008).

Like CLRs, the **Sialic acid-binding immunoglobulin-like lectins** (**Siglecs**) can also recognize pathogens' glycoproteins and glycolipids thus also contributing to the host's innate immune responses. **Siglecs specifically recognize sialic acid-containing glycans** and as mentioned below they also play a relevant role in self recognition (Lock

et al., 2004;Crocker, 2005;Crocker et al., 2007;Kawasaki et al., 2013). The biological and immunological relevance of CLR and Siglec receptors will be discussed in detail in later sections.

DCs can also recognize and internalize microbes and its derivate particles by receptors that bind to opsonins in opsonized ("coated") microbes. **Opsonization of microbes** can occur in two forms: by coating with complement proteins or by binding of antibodies to antigens expressed on their surface. DC recognition of opsonized microbes is thus mainly mediated by **complement receptors** and **Fc receptors** and assures the capture of pathogens that might otherwise evade recognition by other DC receptors (Sedlik et al., 2003;Ben Nasr et al., 2006).

Summarizing, DCs can interact in different ways with microbes, as well as with the host antigens, through a panoply of receptors. This recognition initiates mechanisms that will induce or suppress a specific immune response. DC recognition is thus considered to be of great relevance for the development of a suitable, specific immune outcome, dictating the balance tolerance/reactivity of the developing host-pathogen response.

1.1.3 Dendritic cells-based therapy

The current knowledge of DC immunobiology allowed several biotechnological and pharmaceutical companies to develop DC-based immunotherapies. Applications for DC-based therapy include a plethora of pathologies ranging from infectious and hypersensitivity diseases to malignancies.

The development of moDC cell culture protocols (Romani et al., 1994;Jonuleit et al., 1997) allowing its relatively easy obtention, as well as its pro-inflammatory properties, makes them the preferred DC subset for autologous therapy applications. One strategy is the *ex vivo* upload of moDCs with the antigen to turn them able to efficiently develop an efficient response against the antigen bearer (Caminschi and Shortman, 2012;Matos

et al., 2013;Mintern et al., 2013;Phanse et al., 2013;Yao et al., 2013). The best example of this strategy, with growing media attention, is the **vaccination of cancer patients with DCs loaded with tumor antigens**, the Provenge® (sipuleucel-T) vaccine from Dendreon pharmaceutical company, targeting prostate cancer, being the most prominent example.

Other approaches include the use of specific antibodies targeting DC endocytic receptors that are used to force the upload of specific antigens towards that receptor. Antibodies are also used to block specific receptor-ligand interaction and consequent downstream signaling, counteracting, for instance, the negative immunomodulatory cues of the tumor microenvironment.

DCs have also been studied as vectors for DNA vaccines encoding for antigens (Cao et al., 2013). Viral transduction of DCs not only targets antigens to these cells, but also induces intracellular pathways to modulate the immune response (Liechtenstein et al., 2013).

All these relatively recent drug-niche that exploits DC unique immune potential is proof of reconnaissance of DCs' cornerstone role in the immune system. Nevertheless, DC-based therapies still face several hindrances until their full application, most notably having a relatively low overall efficacy (Dougan and Dranoff, 2009;Draube et al., 2011). The low efficacy is mostly derived from the lack of full knowledge regarding DC biology, including the pathogenesis/tolerance balance and their migratory mechanisms. The identification of novel targets to modulate DC functions are still needed.

1.2 Glycosylation as an immunomodulation target

Glycosylation of cell surface and secreted proteins and lipids occupy the juncture where critical host-host and host-pathogen interactions occur. The role of glycan epitopes in cell-cell and cell-pathogen adhesive events is already well-established, and cell surface glycan structures change rapidly in response to stimulus and inflammatory cues. **Glycosylation is the most frequent modification of proteins and lipids**. The majority of glycans exist as membrane-bound or soluble glycoconjugates. One consequence of this fact is that all cells present at their surface a **glycocalyx**, that is, **the full surface-complex of glycans and glycoconjugates**. The **two main classes of glycoconjugates** are **glycoproteins** and **glycolipids** and their synthesis occurs mainly in (but not limited to) the lumen of the endoplasmic reticulum and in the Golgi apparatus. In glycoproteins, the sugar chain is classified as being ***N*-linked** or ***O*-linked**, depending if the glycosidic moiety is linked to an asparagine (Asn) residue in the protein moiety or to a serine/threonine (Ser/Thr) residue, respectively (Figure 1.4).

The **cell glycocalyx** is the result of the expression of the set of enzymes responsible for the synthesis and/or transfer of monosaccharides or glycosylated structures, i.e., the **glycosyltransferases**. Also critical is the expression of enzymes responsible for the partial removal of glycans or even entire structures from glycosylated molecules, i.e., the **glycosidases**. These two sets of enzymes work in a finely controlled balance during the glycoconjugate synthesis at the Golgi apparatus. Both enzyme types can also be present in plasma membrane or soluble forms, with potentially relevant biological roles as we shall see in sections below (Banerjee, 2012;Hennet, 2012;Rosnoblet et al., 2013).

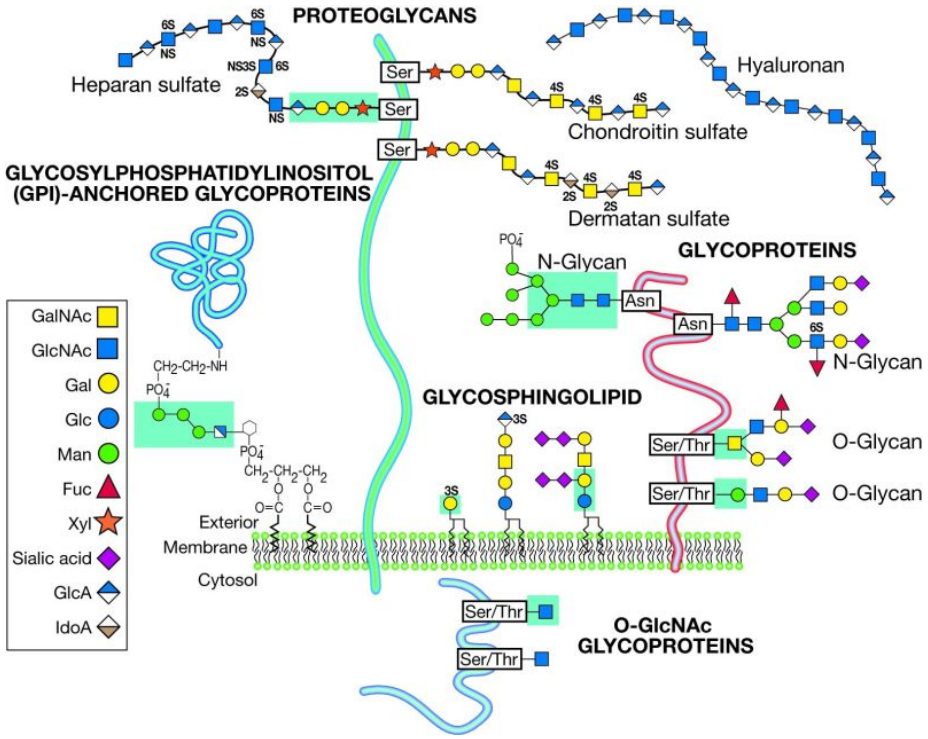


Fig. 1.4 - Common classes of animal glycans. *In Varki A et al. (Ed.),* "Essentials of Glycobiology", *Cold Spring Harbor Laboratory Press, 2nd Edition, 2009.*

Sialic acids are a large family of negatively charged, nine-carbon monosaccharides that are normally found at glycan terminal positions, and one of most common among vertebrates is ***N*-acetylneuraminic acid (Neu5Ac).** On the cell surface, sialic acid residues can be present in *N*- and *O*-glycans in glycoproteins, as well as in gangliosides, i.e., a glycolipid containing one or more residues of sialic acid.

The **sialyltransferases** are a family of twenty glycosyltransferases that catalyze the addition of sialic acids to terminal non-reducing position of the oligosaccharide, transferring the sialic acid from the activated sugar donor **cytidine-monophosphate-Neu5Ac** (**CMP-Neu5Ac)** to different sugar acceptors (Table 1.1) (Varki et al., 2009). Sialyltransferases normally locate at the Golgi apparatus as integral membrane proteins adding sialic acids to glycoconjugates during their synthesis. However, some sialyltransferases are also expressed as soluble enzymes (Jones et al., 2010a) and sialyltransferase activity at the plasma membrane has also been reported in immune

cells (Cabral et al., 2010). Each sialyltransferase presents high selectivity towards its acceptor substrate. *In vivo*, competition between other sialyltransferases and glycosyltransferases' common substrates is observed. As a result, the cell's sialylation status is the dynamic sum of transferase activities, Golgi localization and concentration of activated sugar donors. Sialyltransferases depending on their specificities, can establish **α2,3-, α2,6-, α2,8-linkages** and can be organized in four families depending on linkage specificity and acceptor substrate (Fig. 1.5):

- the **ST3Gal family**, catalyzing the addition of sialic acid to a terminal galactose of ***N*- or *O*-linked glycans and glycolipids in an α2,3-linkage**;
- the **ST6Gal family, α2,6-linking sialic acids to galactose residues of *N*-glycans**;
- **the ST8Sia family**, the only known sialyltransferases promoting the **linkage to another sialic acid residue in *N*- or *O*-glycans**, in a **α2,8-bond**;
- and, finally, **the ST6GalNAc family**, adding sialic acid to terminal ***N*-acetylgalactosamine (GalNAc) residues** of glycoproteins and glycolipids, in an **α2,6-linkage** (Harduin-Lepers et al., 2001).

The overall sialic acid content of a cell is also regulated by the removal of sialic acid residues, catalyzed by the **sialidases**. In humans, four known enzymes fit into this family, also known as **the Neuraminidase family**: **Neu1, Neu2, Neu3 and Neu4**. These sialidases are variedly distributed, with **Neu1 located at the lysosomes and** also expressed **on the surface of diverse types of cells**, **Neu2 at the cytosol**, **Neu3 integrated in the cell membrane** and **Neu4 being an intracellular protein** (Varki et al., 2009). They are all exoglycosidases, i.e., they cleave terminal sialic acids, but have different substrate specificities: **Neu1, Neu2 and Neu4 remove sialic acid residues from glycoproteins**, **Neu2 and Neu4** also cleaves sialic acids from **glycolipids** and **Neu3** preferentially hydrolyzes **gangliosides**.

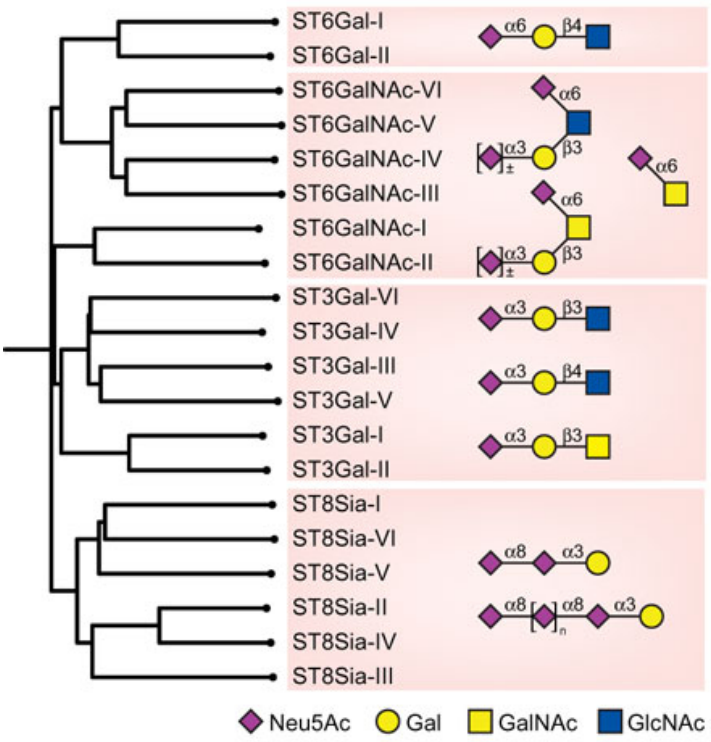


Fig. 1.5 - Structural relationships within the human sialyltransferase family. Homology dendrogram is shown for 20 members of the human sialyltransferase family. Sialoside products produced by each of the four major subfamilies are shown in symbol form. *In Paulson JC and Rademacher C*, "Glycan terminator", *Nature Structural & Molecular Biology 16, 1121 - 1122 (2009)*

A list of human sialyltransferases and sialidases, their expression patterns in DCs, and their preferred acceptor and donor substrates, is shown in Table 1.1 (next page).

Table 1.1 - Human sialyltransferases and sialidases. Preferred substrates and glycan specificity and expression pattern in human dendritic cells are indicated. Data was based on (Bax et al., 2007;Videira et al., 2008;Stamatos et al., 2010;Miyagi et al., 2012).

	Preferred saccharide substrate	**Glycan specificity**	**Dendritic cell expression (cell status)**
Sialyltransferase			
ST3Gal-1	Galβ1,3GalNAc	*O*-glycan	Yes
ST3Gal-2	Galβ1,3GalNAc	*O*-glycan	Yes (mature)
ST3Gal-3	Galβ1,3(4)GlcNAc	*O*-glycan, *N*-glycan	Yes (mature))
ST3Gal-4	Galβ1,4(3)GlcNAc	*N*-glycan, *O*-glycan	Yes (mature)
ST3Gal-5	Galβ1,4GlcCeramide	Glycolipid	Yes
ST3Gal-6	Galβ1,4GlcNAc	*N*-glycan	Yes
ST6Gal-1	Galβ1,4GlcNAc	*N*-glycan	Yes
ST6Gal-2	Galβ1,4GlcNAc	*N*-glycan	No
ST6GalNAc-1	GalNAcα1,O-Ser/Thr Galβ1,3GalNAcα1,O-Ser/Thr	*O*-glycan	No
ST6GalNAc-2	Galβ1,3GalNAcα1, O-Ser/Thr	*O*-glycans	Yes
ST6GalNAc-3	Siaα2,3Galβ1,3GalNAc	*O*-glycan	Yes (undetermined)
ST6GalNAc-4	Siaα2,3Galβ1,3GalNAc	*O*-glycan	Yes
ST6GalNAc-5	GM1b	Glycolipid	No
ST6GalNAc-6	All α-series gangliosides	Glycolipid	Yes
ST8Sia-1	Siaα2,3Galβ1,4Glc-Ceramide	Glycolipid	No
ST8Sia-2	Siaα2,3Galβ1,4GlcNAc	*N*-glycan on NCAM	No
ST8Sia-3	Siaα2,3Galβ1,4GlcNAc	*N*-glycan on NCAM	No
ST8Sia-4	$(Sialα2,8)_nSiaα2,3Galβ1$-R	*N*-glycan on NCAM	Yes
ST8Sia-5	GM1b, GT1b, GD1a, GD3	Glycolipid	No
ST8Sia-6	Siaα2,3(6)Gal	Sialic acid on *O*-glycan	Unknown
Sialidases			
Neu1	Siaα2,3 Siaα2,6	Oligosaccharides Glycopeptides	Yes
Neu2	Siaα2,3 Siaα2,6	Oligosaccharides Glycopeptides Gangliosides	No
Neu3	Siaα2,3 Siaα2,6	Gangliosides	Yes (mature)
Neu4	Siaα2,3 Siaα2,6	Oligosaccharides Glycopeptides including mucins Gangliosides	Yes

1.3 Sialylation and modulation of the immune response

The terminal position occupied by sialic acids on membrane and extracellular glycans puts them on the frontline during leukocyte communication and overall immune response. Sialic acids, on an immune perspective, can function in two (seemingly contradictory) major ways: as **biological masks** and as **recognizable cell patterns/markers** (Varki and Gagneux, 2012).

As a biological mask, **sialic acid helps shield host cells from pathogen and/or host recognition** (Kelm and Schauer, 1997)**.** During acute phase inflammation a rise in soluble and circulatory forms of sialyltransferases occurs, leading to an increase in soluble and cell surface sialic acid content. **Higher sialic acid is thus part of the acute phase response** and it seems to protect cells against pathogens, and may **help the immune system distinguishing 'self' from 'non-self' antigens** (Jamieson et al., 1993). ST6Gal-1 is an example of sialyltransferase whose soluble expression is upregulated during an acute phase response and its expression has been used by some authors as a serological clinical marker for inflammatory conditions (Kaplan et al., 1983;Jamieson et al., 1987;Yasukawa et al., 2005).

Non-sialylated glycans are recognized by specific lectins, and the addition of sialic acid to its terminal position may block lectin binding and associated functions. As an example, the presence of **α2,6-linked sialic acids on *N*-glycans blocks recognition by galectins** (Zhuo and Bellis, 2011). Galectins are a family of β-galactoside-binding lectins that regulate a broad range of cell behaviors, such as cell adhesion, migration, proliferation, differentiation, transformation, apoptosis, angiogenesis, and immune responses (Hughes, 2001;Leffler et al., 2004;Hsu et al., 2006;Nakahara and Raz, 2006;Erbacher et al., 2009).

However, **sialic acid masking can also be used by pathogens** as a **mimicry tactic** in order to evade the immune system. This is the case of some *Trypanosoma* spp. that

have **mutated ST3Gal sialyltransferases acting as trans-sialidases**, transferring the host's sialic acid to coat themselves in order to evade host recognition (Paris et al., 2005;Freire-de-Lima et al., 2012).

1.3.1 Sialic acid-recognition in immune processes

Sialic acids or sialylated glycans can be recognized by several cell surface receptors, such as the previously mentioned CLRs and Siglecs (Sperandio, 2006;Crocker et al., 2007).

Siglecs are sialic acid-recognizing proteins that, albeit structurally similar, are commonly organized in two categories: ***i)*** one comprises **the CD22 family** (including CD22 (or Siglec-2), sialoadhesin (or Siglec-1), myelin-associated glycoprotein (MAG or Siglec-4) and Siglec-15; and ***ii)*** **the CD33-related family** comprising CD33 (or Siglec 3), Siglec-5, -11, -14 and -16 in humans, all chiefly expressed in myeloid and lymphoid cells (Pillai et al., 2012;Varki and Gagneux, 2012). Siglecs recognize and bind ligands present not only in other cells (viz. in *trans*) but also on the same cell (in *cis*). Many Siglecs present one or two **intracellular tyrosine-based inhibitory motifs (ITIMs)** immunoreceptors, classically described as being involved in signaling to regulation-inducing pathways, or **intracellular tyrosine-based activation motifs (ITAMs)**, involved in the initiation of activation signaling pathways (Flaswinkel et al., 1995;Isakov, 1997). Hence, Siglecs have a decisive role in regulating, either positively or negatively, immune responses such as inflammation or tissue damage by actively discriminating between **self-associated molecular patterns (SAMPs)** and **PAMPs** (Crocker et al., 2007;Crocker and Redelinghuys, 2008;Liu et al., 2009;Varki and Gagneux, 2012). We will discuss the functional relevance of these receptor families in DC-related aspects, later on, in section 2.4.1.2.

Studies using mice deficient for selected α2,3- and α2,6-sialyltransferases have provided evidence confirming the importance of sialic acid in immune processes (Hennet et al., 1998;Priatel et al., 2000b;Moody et al., 2003). **ST6Gal-1$^{-/-}$ mice** have **impaired humoral immune response**, characterized by **reduced levels of circulating and cell-surface IgM, impaired B cell proliferation in response to various activation signals, and impaired antibody production following contact with antigens** (Hennet et al., 1998). CD22, one of the first described Siglecs (Powell et al., 1993), was later shown to recognize ST6Gal-1-mediated glycans, **functionally regulating several B cell functions and survival mechanisms** (Ghosh et al., 2006). Other ST6Gal-1 KO mice studies have also revealed that **soluble forms of ST6Gal-1 have a relevant role in myelopoiesis during acute inflammation**, namely, by limiting it, thus avoiding uncontrolled excessive neutrophilic and eosinophilic inflammatory responses (Nasirikenari et al., 2006;Jones et al., 2010a;Nasirikenari et al., 2010).

These are only few examples on how sialic acids influence immune-relevant processes. Other examples include roles in host-pathogen interactions, regulation /modulation of the acute phase response and influence in the progression and differentiation of human malignancies.

1.4 Dendritic cells and glycosylation

As previously mentioned, DCs play a role of great relevance in the immune system. The characterization of the DC's 'glycome' – the whole set of glycosylation features ('sialome' included) –, its functional impact on the DCs immunobiology and, of course, on the immune system has been a work in progress. Glycosylation of DCs has consistently being proven to be a very relevant feature, similar to what occurs with other immune cells. The most recognized feature is probably DC adhesion where selectin ligands of glycosidic nature are deemed crucial in the migration process. But, nonetheless, it is also known that glycosylation has a relevant role in the remainder immune processes as well, especially in innate recognition and DC activation, where the boundary condition of the DC glycocalyx makes glycans key players in these processes. There are many questions still open, such as DC migration to specific sites, with predictable impact in many potential clinical applications, as is the case of DC-based vaccines.

1.4.1 Sialylation in dendritic cells

The interest in studying the role of sialylation in DCs, resulted from the large focus on DC's immunobiology and also from the growing scientific interest in glycobiology (including sialic acids). The role of sialylation has been mostly studied in moDCs (inflammatory DCs) for the reasons previously mentioned (see section 1.1.1.2.1).

Jenner and coworkers stated that **immature moDCs present a high sialylation content**, namely α2,3- and α2,6-sialylated glycoproteins, when compared to its monocyte precursors. These results, also corroborated by our work, will be explained in further detail below (Jenner et al., 2006;Videira et al., 2008). Both sialyltransferases and sialidases undergo significant gene expression variation during differentiation from

monocyte to moDC (Jenner et al., 2006;Videira et al., 2008;Trottein et al., 2009;Stamatos et al., 2010), namely, a **significant upregulation of the ST3Gal-1 and ST6Gal-1 genes** occurs during moDCs' differentiation. The up-regulation correlates with increases of measurable enzymatic activities attributable to these two enzymes and the increased degree of α2,3- and α2,6-sialylation (Videira et al., 2008) during myeloid lineage-committed differentiation. The data implicate these two sialyltransferases as the major regulators in the biosynthesis of α2,3- and α2,6-linked sialic acid-containing glycan structures specific for moDCs. Interestingly, the membrane sialidases, **Neu1 and Neu3 are also significantly upregulated** during DC differentiation (Stamatos et al., 2010), probably acting in more specific cell glycans.

moDCs maturation is also accompanied by an increase in α2,3-sialylation and, contrasting, a decrease of α2,6-sialylation that is likewise correlated with changes in the sialyltransferase and sialidase activities (Jenner et al., 2006;Bax et al., 2007;Videira et al., 2008). Moreover, the reported sialylation variations are stimulus-dependent processes (Videira et al., 2008). These observations are summarized on the present chapter's Table 1.1, on section 1.2.

While the functional impact of these observed sialic acid changes remains to be elucidated, there is already some indication supporting the biologic relevance, and it will be discussed later on.

1.4.1.1 Sialic acid-recognizing dendritic cell receptors

One of the most immediate aspects of sialic acid-containing glycans expressed by DCs is being the target of receptors. In this aspect, **Siglecs** are the largest and most relevant family.

Recognition of DCs' sialylated glycans has some known functional implications: e.g., inhibitory T cell Siglecs recognizing high α2,6-sialic acid content of immature and tolerogenic DCs as part of a host-tolerance inducing mechanism (Jenner et al., 2006), or the observed increased binding of **Siglecs-1, -2 and -7** correlating with the higher sialic acid content of mature DCs (Bax et al., 2007). This gathered evidence hints at an even more promising, relevant role of Siglec-mediated immunobiological processes involving DCs and other leukocytes, but still to be unraveled and requiring, thus, further studies.

Besides being recognized by Siglecs through their expression of glycans, DCs also express Siglecs that enable them to also recognize sialylated structures on self or on neighboring cell surfaces. **MoDCs and blood-circulating DCs (namely plasmacytoid, CD1a$^+$ and CD141$^+$ DCs** (Hemont et al., 2013)) **express Siglec-1, -2, -3, -5, -7, -9, -10, -14 and -15** (Lock et al., 2004;Bax et al., 2007;Crocker and Redelinghuys, 2008;Crocker et al., 2012), with plasmacytoid DCs having a more restricted pattern and apparently only expressing **Siglec-5** (Lock et al., 2004). Siglecs, with the exception of Siglec-14 and -15, expressed by DCs present immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytosolic portion and are therefore mainly involved in inhibiting activation signals and have an immunoregulatory function (Crocker, 2005;Crocker et al., 2007).

The concentration of sialic acids on surfaces of human cells is very high; for example, Stamatos and colleagues estimated that DCs had 8.9 nmoles of sialic acid per $5x10^6$ cells, which correspond to nearly 10^{18} sialic acid molecules per cell (Stamatos et al., 2010). Therefore, it is possible that the majority of Siglecs expressed at DC surface bind ***in cis*, i.e., to sialic acids at their own cell surface**. Due to short length of Siglecs compared with other cell surface proteins, the ***cis* interaction normally has primacy over the *trans* interactions** – the only exception being sialoadhesin (CD22), which has an extended structure, projecting their binding site away from plasma membrane and being therefore involved in trans interactions (Freeman et al., 1995).

Siglec interactions *in cis* can be released as a result of sialidase activity, expressed either **extrinsically** – for instance from pathogens –, or **intrinsically** – due to the activity of endogenous sialidases (Crocker, 2002;2005).

Siglecs potentially play a largely relevant role **in host-tolerance mechanisms**. (Lock et al., 2004;Bax et al., 2007;Santos et al., 2008). Chen and co-workers reported **Siglec-10** as involved in helping to distinguish TLR-recognized, **danger-associated molecular patterns (DAMPs)** – generated during cell/tissue damage or even regular cell cycle – from PAMPs, thus controlling inflammation (Chen et al., 2009). There are known examples of T cell activation where DC Siglecs have a relevant role in inducing T_h1 and T_h2 responses, as is the case of DC **Siglecs-1** and **-7 *trans* recognition of α2,3-sialic acids and α2,8-polisialic acids**, respectively, in mimicked GM1a and GD1a (Siglec-1 recognized) and GD1c (Siglec-7 recognition) gangliosides included in *Campylobacter jejuni*'s lipopolysaccharides (Bax et al., 2011).

CD33-related Siglecs can function as endocytic receptors that are important in the clearance of sialylated antigens. On the other hand, many pathogens are able to express appropriate sialic acids themselves (Jones et al., 2003;Avril et al., 2006;Carlin et al., 2007;Khatua et al., 2010). Pathogen's sialic acids may interfere with DC functions, such as endocytosis (Lock et al., 2004;Biedermann et al., 2007) thus helping DCs to internalize and further present pathogen's antigens. This, however, may also open an opportunity window for pathogens to modulate DCs' immune functions (by binding to immunoregulating Siglecs) or even use DCs as vectors (i.e. "Trojan horses") for infection of other immune system cells, e.g. the Human Immunodeficiency Virus (HIV) using Siglec-1 as a gateway-receptor for DC entry and posterior transmission to $CD4^+$ T cells (Izquierdo-Useros et al., 2012). However, a safety mechanism may be present: Siglec-15 can act as an activation receptor balancing the negative signaling triggered after recognition of sialylated pathogens (viz., enveloped viruses) through inhibitory Siglecs (Crocker and Redelinghuys, 2008).

1.4.1.2 Dendritic cell sialylation and endocytosis

The sialic acid's role on endocytosis has long been studied on the perspective of the pathogen. Adding to the already referred trans-sialidase bearing *T. cruzi* parasite, it is also known that several bacteria developed sialic acid-masking mechanisms in order to escape immune surveillance and/or response (Severi et al., 2007). As part of the work presented on this thesis, recent discoveries, however, hinted that sialic acid's role in these immune processes surpasses its simple antigen status, with a functional impact on the innate immune phase cells as well, like DCs.

1.4.1.3 Sialylation and dendritic cell migration

DC migration includes both **DC recruitment to non-lymphoid tissue** and **homing to lymphoid organs**.

DCs residing within tissues may respond to pro-inflammatory cytokines and pathogens hence triggering maturation. DCs then migrate to lymphoid tissues via afferent lymphatic vessels, wherein they activate antigen-responsive T cells. More rarely, immature and mature DCs may also enter the blood, and from there, disseminate to non-lymphoid and lymphoid organs, thereafter returning to blood, thus undergoing cycles of recirculation. DCs have complex trafficking routes, allowing for their dynamic reassortment, making the most of their capacity to uptake antigens and to encounter T cells to present antigens and activate them.

While, generally, the migratory processes are based upon mechanisms like adhesion and chemotaxis, some processes still show their own particularities. The **extravasation of blood DCs** to any tissue **involves DC adhesion to endothelium** and is dependent of **selectin interactions with sialofucosylated glycans**. The role of certain sialylated

glycans as selectin ligands is one of the most recognized functions of sialic acid in the context of leukocyte recruitment (Sperandio et al., 2009).

Selectins are CLRs expressed by **p**latelets, **e**ndothelium or **l**eukocytes, hence taking their name: **P-, E- or L-selectins**, although **endothelial cells also express P-selectins**.

All selectins recognize **selectin ligands**, with the sialic acid- and fucose-containing tetrasaccharide **sialyl-Lewis X antigen** (**sLe^x)** (Neu5Acα2,3Galβ1,4[Fucα1,3]GlcNAc-) being the most studied structure. **Selectin ligands are expressed in most circulating immune cells and some endothelial cells during inflammation** (Fig. 1.6). They mediate the rolling and tethering phase of cell transmigration over the endothelial cell surface (McEver, 2002).

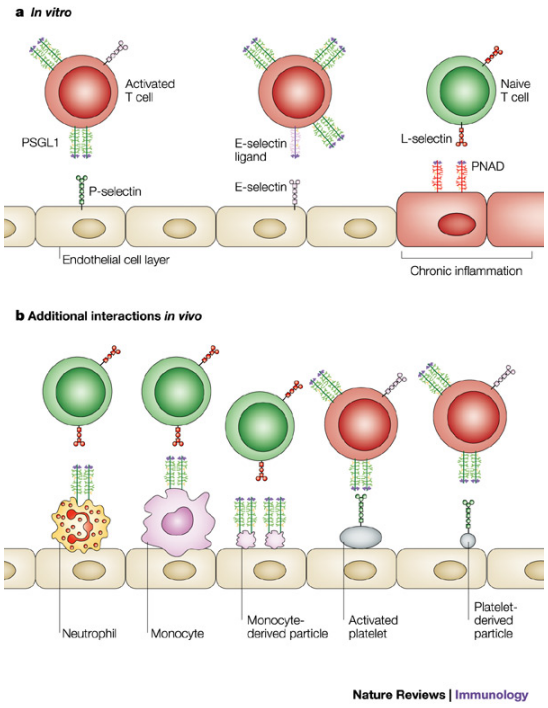


Fig. 1.6 - Selectin-dependent interactions. *In Ley K. and Kansas G.S.*, "Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation", *Nature Reviews Immunology, (4), 2004*.

sLe^x expression is well characterized in neutrophils and lymphocytes (Patel et al., 1995;Sperandio, 2006) but only relatively approached in DC (Robert et al., 1999a;Pendl et al., 2002;Julien et al., 2007;Silva et al., 2011). Recently, it was found that **moDCs also express functional selectin ligands**, based on their observations of moDC tethering

and rolling over a P-, E- and L-selectin immobilized surface (Silva et al., 2011). They observed decreasing tethering affinities (by decreasing order) towards **P-selectin, E-selectin and L-selectin**, with similar lower rolling velocity on P- and E-selectins and the largest rolling velocity observed over L-selectins. These findings were in line with other studies using blood DCs and $CD34^+$-derived DCs (Robert et al., 1999a;Urzainqui et al., 2007). Furthermore, the use of anti-sLe^x antibodies on the rolling studies resulted in a significant binding inhibition, definitely proving that sLe^x mediates the moDCs-selectin binding (Silva et al., 2011).

In order to properly function as a selectin ligand, sLe^x must be expressed in carrier glycoproteins or glycolipids (Foxall et al., 1992;Marth and Grewal, 2008). **The only described sLe^x carrier-protein described in moDCs is the P-selectin glycoprotein ligand-1 (PSGL-1)** (Julien et al., 2007), a mucin-like glycoprotein, present in the microvilli of most leukocytes (Laszik et al., 1996;Kieffer et al., 2001) (Figure 2.7). In DCs, **sLe^x-decorating PSGL-1 is the sole ligand for P-selectin, with significant less affinity towards L-selectin and being indifferent for E-selectin binding** (Silva et al., 2011).

Nevertheless, sialic acids also participate in the chemokine receptor-mediated firm arrest, as well as in β_1 integrins function (Semel et al., 2002;Frommhold et al., 2008;Woodard-Grice et al., 2008). There is also evidence concerning the chemokine-mediated migration to the lymph nodes. It was recently reported that ST8Sia-IV-dependent polisialylation of neuropilin-2 seems to be relevant for CCL21-driven migration towards lymph nodes (Rey-Gallardo et al., 2011). Other report claims that ST3Gal-4 is not relevant for CCR7-dependent DC homing, in the mouse model (Frommhold et al., 2008). Nevertheless, the large body of evidence points to α2,3-sialylation as the prominent sialylation form involved in DC migration.

In the work for this thesis, we sought to contribute to this issue by studying DC homing in ST6Gal-1-deficient mice. As reported in section 4.3, the results suggest that DC

migration towards draining lymph nodes is significantly impaired, suggesting a previously unknown role for α2,6-sialylated *N*-glycans in DC homing.

DC mobility is a crucial step still in need of better elucidation. Most of the clinically efficacy of DC immunotherapy relies on the migration ability of these cells. In *ex vivo* generated DC vaccines, it is estimated that only 1-2% of total administered DCs reach secondary lymphatic organs (Ridolfi et al., 2004). Therefore, the majority of *ex vivo* generated DCs are inefficient because they do not meet T cells. Thus, understanding DC migration should be regarded as important to find means to improve DC immunotherapy.

1.4.1.4 Sialic acid in dendritic cell-T cell interactions.

The ultimate function of DC immunobiology is the DC-T cell interaction, whereupon DCs present the uptaken, processed antigens to T cells, to initiate a specific, long-lasting immune response.

DCs' sialic acid-containing glycans have been shown to negatively influence T cell priming, most likely by interference on MHC-mediated antigen presentation and co-stimulation (Boog et al., 1989;Dubsky et al., 2005). However, one should not discard a synergistic effect with enhanced protein-protein interaction due to the absence of the negatively charged sialic acid (Boog et al., 1989), leading to enhanced inter cellular interactions.

Reinforcing these results, others have observed that endogenous sialidase activity also promotes cytokine production by moDCs, in what was attributable to the action of Neu3 upregulation during moDC differentiation (Stamatos et al., 2010). Interestingly, tolerogenic, immature moDCs present high sialic acid content (Jenner et al., 2006) and it has been hypothesized that, host tolerance induction by DCs could be a Siglec-mediated process (Jenner et al., 2006).

The contribution to this issue, herein presented, is solid evidence that **sialidase-treated moDCs are able to prime T cells and to induce proliferation more efficiently than fully sialylated moDCs.** This effect could be attributable to the increased maturation by sialidase-treated moDCs but the effect of the verified upregulation of a set of pro-inflammatory, T_h1 profile-inducing cytokine expression (viz., **IL-1α, -6, -12 and TNF-α**) in sialidase-treated moDCs (with subsequent IFN-γ secretion) accounting for the observed increased priming is not excluded.

Taken together, this evidence reminds that DC sialylation has implications in their interaction with T cells and it should be considered to fine tune DC-based therapy either pathology-treating or tolerance-inducing.

1.4.2 Dendritic cell glycan-recognition of tumors

Although not covered by the work within this thesis, the relevance of DCs functions in onco-immunology should be mentioned.

Recognition of tumor antigens by DCs elicit a maturation response on DCs that is critical to the initiation of anti-tumor immune responses.

The immune potency of such response depends on many factors, including, but not limited to, the antigen. Tumor cells are able to evade immune responses by creating a tolerance-inducing microenvironment, which includes the secretion of inhibitory factors and activation of immunosuppressant intracellular pathways in the immune cells which leads to defective maturation of DCs (Banchereau and Steinman, 1998b;Rabinovich et al., 2007;Condamine and Gabrilovich, 2011). Furthermore, matured DCs present flaws in their tumor antigen-presenting capacity and a faulty ability to prompt T cell responses, thus defective in generating effective anti-tumoral solutions (Rabinovich et al., 2007;Benencia et al., 2012).

Aberrant glycosylation is a hallmark of cancer cells and the aberrantly glycosylated proteins are shed into the bodily (serum, urine, pleural effusions, etc). Altered glycosylation in tumor cells can result from a decrease or increase of expression of certain glycan structures, the appearance of truncated structures, or even novel structures. Upregulation and/or downregulation of specific glycosyltransferases are often regarded as drivers for these changes. Tumor-associated carbohydrate (TAC) structures allow tumor cells to invade and metastasize or to evade the immune system. For instance, sialyl-Tn antigen (STn) expression by cancer cells is associated with many malignant features such as invasiveness and cell differentiation (Pinho et al., 2007;Julien et al., 2012). Moreover, STn^+-bearing MUC1 mucins inhibited DC maturation and modulate DCs towards IL-10^{high} IL-12^{low} antigen presenting cells (Monti et al., 2004). Recently, Carrascal et al. have shown that DCs pulsed with antigens derived from STn^+ cancer cells induced T cells to anergy, displaying a FoxP3^{high} IFN-γ^{low} phenotype, in a mechanism likely to involve STn^+ MUC1 and CD44 antigens (Carrascal et al., 2014).

How TAC contributes to immune evasion depends on their recognition by DC receptors. The few available studies point, so far, to **the CLRs, MGL-1, DC-SIGN and Siglecs receptors as being relevant in TAC recognition by DCs** (van Gisbergen et al., 2005;Saeland et al., 2007). MGL-1 is highly expressed in immature, tolerogenic DCs and shown to interact with the tumor-associated Tn antigen-bearing forms of MUC1 (Napoletano et al., 2007). The latter is also expressed by immature DCs and recognizes Le^x and Lewis Y (Le^y) glycoantigens in a carcinoembrionic antigen-context expressed in colorectal carcinoma (Fig. 1.7). Besides these receptors, the observed involvement of DCs' Siglecs (such as Siglec-3 and -9) could help justifying the frequent tolerance-induction mechanisms via recognition of sialylated antigens (e.g. sialyl T and sLe^a), usually overexpressed by tumor cells. These receptors could send inhibitory intracellular signals from their ITIM motifs thus preventing DCs from differentiating (by inducing apoptosis of their precursors) or maturating, keeping them in a tolerance-inducing state

with concomitant upregulated anti-inflammatory cytokine expression, downregulated pro-inflammatory cytokine expression and reduced antigen presenting capability (Rughetti et al., 2005;Ishida et al., 2008;Ohta et al., 2010).

It is now evident that TAC and, in particular, sialic acid-containing TAC influences tumor progression. **DCs become tolerogenic after recognition of TAC, favoring tumor progression and being generally associated with bad prognosis**. The collected evidence regarding the glycan influence on anti-carcinogenic immune processes should be, therefore, seriously considered whenever DC-based immunotherapies against specific malignancies are available.

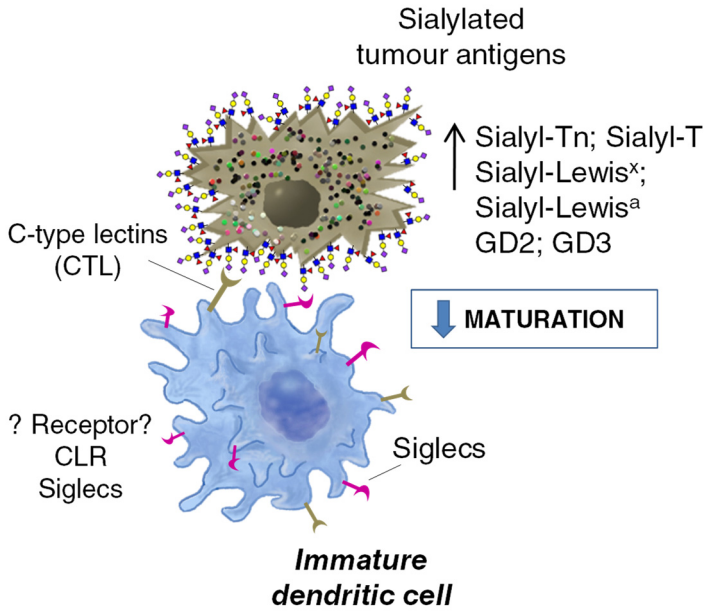


Fig. 1.7 – Sialic acid-modulated DC-tumor cell interactions generally elicit tolerance responses, favoring unchecked tumor progression.

1.5 Concluding remarks

DCs are pivotal, key players of the immune system, influencing and defining the immune response and outcome upon antigen uptake and the induction of adaptive immune responses. DCs are important sentinels due to their special mechanisms for antigen capture and processing, and a unique capacity to migrate to defined sites in lymphoid organs, to meet T cells. They also undergo rapid maturation in response to a variety of stimuli that act in receptors such as PAMP binding in Toll-like receptor or cytokines in cytokine receptors. Due to their role in dictating adaptive immune responses, DCs are now being used as cell based vaccines to treat cancer patients. However, many of their mechanisms are still poorly understood and the efficacy of DC-based immunotherapeutic at the clinical setting is limited.

The importance of glycosylation and, in particular, sialic acid in biological processes is being increasingly acknowledged. Being at the terminal position of many glycans, sialic acids are located in a privileged position and can be recognized by many receptors or may interfere with many ligand-receptor interactions. Thus, sialic acids can (potentially) play an essential role in modulating many of the DC functions.

Understanding the DC-sialylation binomial can prove to be the definite element to achieve better forms of immunotherapy still to be clinically developed. Sialic acid modifications on DCs, through Glycoengineering may be very promising tool to fine tune DC functions and can help surpass the current hindrances affecting the efficacy of DC-based therapy.

Section 2

OBJECTIVES

Dendritic cells (DC) play a central role in the interface between innate and adaptive immune responses. Although the DC subset comprises several subtypes, all DC subtypes' main, immunologically relevant functions can be summed up in three phases: 1) antigen uptake at peripheral tissues and/or secondary lymphoid organs, 2) maturation and homing to the regional draining lymphatic organs, and 3) antigen presentation/T cell priming. The elucidation of the mechanisms that modulate their function is important for understanding the immune response, identification of therapeutic targets and development of novel immunotherapies.

Sialic acid moieties play a relevant role in modulation of immune functions, such as antigen recognition, cell adhesion and migration, among others functions, due to their terminal position at cell surface glycans.

Considering the high sialic acid content of DCs, **our primary hypothesis is that sialic acid has an impact in DC functions, such as antigen uptake, maturation, homing to lymph nodes and antigen presentation to T cells**. As a matter of fact, sialic acid moieties have been shown to modulate important functions of lymphocytes (Hennet et al., 1998;Priatel et al., 2000a), but their role in DC functions were unknown.

In this thesis, to test this hypothesis, **the following specific objectives were established**:

1 – To characterize the sialylation phenotype of DCs in their immature and mature states.

2 - To analyze the expression of sialyltransferases responsible for DC sialylation and enzymatically determine their activity, during the differentiation and maturation phases;

3 – To investigate the influence of α2,3- and α2,6-sialylation of DCs in the tissue distributions of these cells in the mouse;

4 – To determine how does sialylation affect the antigen uptake, maturation, homing and capacity to stimulate T cells by DCs.

5 – To contribute to the assessment of the underlying intracellular signalling processes affected by sialic acid modulation, and the involved sialylated receptors;

Ultimately, this work aims to understand whether the engineering of cell surface sialylation, mediated by individual sialidases or sialyltransferases, is a possible tool to fine tune the main DC functions, with particular significance to DC-based therapies.

Section 3

MATERIALS AND METHODS

3.1 moDC and BMDCs differentiation and maturation

3.1.1 Human monocytes' separation

Differentiation of monocytes to monocyte derived DCs (moDCs) is currently a widely used technique to obtain large numbers of DCs. Monocyte precursors are normally present in the average healthy adult, at a steady-state concentration of 200-800×10^3 cells/mL (2-10% of blood mononuclear cells). In this study, we use blood leukocyte concentrates obtained from whole blood donations, to obtain an enough amount of monocytes and hence to obtain a reasonable amount of moDCs.

Mononuclear cells were isolated by a Ficoll gradient centrifugation, from blood 'buffy coats' of healthy volunteers provided by the Portuguese Blood Institute. For this, blood from the buffy coats was diluted 2 parts blood to 3 parts of Phosphate Buffer Saline (PBS) (v/v), pH 7.4 and then centrifuged at 1200×*g* (2500 r.p.m. on a Heraeus Sepatech model Megafuge 1.0R centrifuge equipped with a BS4402/A basculant rotor) for 20 min in order to obtain a rough mononuclear leukocyte concentrate. After dilution in 30 mL phosphate buffer saline (PBS), the mononuclear cell concentrates were separated by Ficoll gradient (Biochrom AG), at a proportion of cell suspension to Ficoll solution of 5:3, centrifuging at 1200×*g*, for 20 min. After centrifugation, a clear white ring formed by **p**eripheral **b**lood **m**ononuclear **c**ells (PBMCs) was carefully removed and gently washed twice with PBS in order to remove debris and contaminating erythrocytes and platelets.

PBMCs were then magnetically sorted using CD14 magnetic Microbeads (Miltenyi Biotech). PBMC cell number was determined and accordingly ressuspended in ice-cold Beads buffer (PBS pH 7.2, 0.5% bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic acid (EDTA)), at a volume fourfold the volume of $CD14^-$ magnetic Microbeads to Beads buffer, calculated according to the following formula:

$$\mathrm{V}_{CD14\ MicroBeads}\ (\mu\mathrm{l}) = \frac{\text{Cell number} \times 20\ \mu\mathrm{l}}{3 \times 10^7\ \text{cells}}$$

This PBMC + CD14 Microbeads suspension was left incubating for 15 minutes, at 4°C, after which cells were washed by adding Beads buffer at a proportion of 1 mL per 10^7 PBMCs and centrifuging 500×*g* for 5 minutes. The washed PBMC/CD14 Microbeads mixture was ressuspended in 500 µl ice-cold Beads buffer per 10^8 PBMCs. In parallel, the magnetic cell sorting column (LS columns, Miltenyi Biotech) to be used was prepared by washing with 3 mL Beads buffer. PBMC/CD14 Microbeads suspension was afterwards applied into the column already mounted in the magnet (accounting for $CD14^+$ cell selection), following a 3 × 3 mL ice-cold Beads buffer wash removal of $CD14^-$ fraction. This fraction was discarded except when autologous lymphocytes were needed for the T-cell priming assay. Elution of $CD14^+$ cells was then performed by removing the column from the magnet, filling it with 5 mL ice-cold Beads buffer and, with the help of the supplied plunger, flushing the cells out of the column. The hence separated $CD14^+$ cells were then counted and prepared for culture.

3.1.2 DC differentiation and maturation

The $CD14^+$ cells, after phenotypical confirmation by flow cytometry (see '*Flow cytometry'* subsection below), were then cultured in **complete culture medium**, namely, RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 100 µg/mL penicillin/ streptomycin (Gibco BRL) and 50 µM 2-mercaptoetanol and 10% fetal calf serum (FCS) (Sigma-Aldrich Ltd.) for 6 days in the presence of 1,000 U/mL human recombinant IL-4 and GM-CSF (R&D Systems) to give rise to immature moDC.

The moDC maturation was induced at 6th day culture, by the addition of 5 µg/mL of LPS (Sigma –Aldrich Ltd.) or 1,000 U/mL of IL-1β, IFN-γ (Sigma-Aldrich) or TNF-α (R&D Systems).

3.1.3 Flow cytometry analysis of antibody- and lectin-cell staining of moDCs

Staining with **F**luorescein **i**so**t**hio**c**yanate (**FITC**), **p**hyco**e**rythrin (**PE**) or **PE-Cy5**-conjugated human antibodies against CD14 (M5E2), anti-CD80 (L307.4), CD86 (Fun-1; IT2.2), HLA-DR (L243) (BD Pharmingen), anti-HLA-ABC (W6/32) (Dako Glostrup) and BDCA-1 (AD5-8E7) (Miltenyi Biotec) was used to monitor monocyte isolation, moDC differentiation and to assess maturation levels.

As a standard procedure, 10^5 cells were washed, ressuspended in 100 µl serum free RPMI 1640 medium and incubated with 10 µl of the selected antibodies for 15 min at room temperature, in the dark.

For the analysis of the moDC maturation phenotype after endocytosis, 2×10^5 cells were first incubated with 0.1 mg/mL of FITC-conjugated ovalbumin, in RPMI 1640 + 10% fetal calf serum, for 1h, at 4°C or 37°C. Following incubation, moDCs were treated or mock-treated with sialidase as described below (subsection *Sialidase treatment*). Half of the cells were reserved for RNA extraction and real-time polymerase chain reaction (PCR). The remaining cells were washed with FACSFlow® (Becton-Dickinson) stained with antibodies against maturation markers (HLA-DR, HLA-ABC and CD80/86) and analyzed by flow cytometry.

On all procedures, after the staining step, cells were washed twice with, and ressuspended in, FACSFlow®. Acquisition and analysis proceeded in a FACSCalibur® flow cytometer (Becton-Dickinson Instruments) using CellQuest® software (Becton Dickinson) and/or INFINICYT® software (Cytognos).

For the lectin staining, 10^5 cells were washed, ressuspended in serum free RPMI medium and incubated with 50 µg/mL of FITC-lectins (extracted from *Maackia amurensis* (MAA), *Sambucus nigra* (SNA) or *Arachis hypogaea* (PNA) species) (EY Labs) for 15 min, at room temperature in the dark. Cells were then washed twice, ressuspended in FACSFlow® supplemented with 0.1% BSA and acquired by flow cytometry.

3.1.4 Animals

ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ mice were obtained from the Consortium For Functional Glycomics and originally developed by Priatel et al. (Priatel et al., 2000a) and Hennet et al. (Hennet et al., 1998), respectively. C57BL/6 (wild-type (WT)) mice (Jackson Laboratories) were purchased to Jackson Laboratories or authorized resellers.

Mice were used at 10–12 weeks of age, with C57BL/6 animals used as controls. All handling of animals was previously approved by the Ethics Committee of the Medical Sciences Faculty of Lisbon and the Institutional Animal Care and Use Committee of Roswell Park Cancer Institute of Buffalo, NY, USA, under the supervision of officially recognized animal handlers. ST3Gal-1$^{-/-}$, ST6Gal-1$^{-/-}$ and WT mice were euthanized by asphyxiation with CO_2.

3.1.5 Murine lymphoid tissue-resident and blood DCs extraction

Lymphoid tissue-resident DCs were collected from mice in the following procedure: after lymph nodes removal, DCs were obtained by flushing said lymph nodes with culture medium and mechanically disrupting the lymphoid tissue on a 70 μm cell strainer with the help of a sterilized syringe plunger. In the extraction of adoptively transferred BMDCs (see below), the culture medium was supplemented with 2.5 mg/mL collagenase D from *Clostridium histolyticum* (Roche Diagnostics).

Spleens were also removed, its contents flushed and finely minced. A 20 min, 37°C incubation with collagenase D at 1 mg/mL, followed by an erythrocyte lysis, using a Tris-HCl 0.17 M, NH_4Cl 0.16 M hypotonic solution, for 5 min at room temperature, were finally performed.

The blood samples were collected by cardiac puncture into tubes containing an EDTA solution as anticoagulant. To this suspension, a hypotonic erythrocyte lysis solution was added in order to prepare the cell suspension for flow cytometry acquisition.

3.1.6 Bone marrow-derived DCs

BMDCs were obtained mainly as described previously by Lutz et al. and Inaba et al. (Inaba et al., 1992;Lutz et al., 1999), with some modifications. Briefly, the bone marrow was flushed from the mice tibiae and femurs to a 10 mm Petri dish with the help of a syringe filled with complete cell culture medium (RPMI-Glutamax-I® supplemented with 5% FBS (v/v), 50 µg/mL gentamicin sulphate (Cellgro), 50 µM 2-mercaptoethanol (Invitrogen)) and a 1/2G needle. The resulting bone marrow suspension was strained with 40 or 70 µm strainers (BD Falcon) and exposed to hypo-osmotic erythrocyte lysis buffer as described above. The bone marrow cells were then counted and cultured at 5×10^5 cells/mL on 6-well plates, in complete culture medium supplemented with 10 ng/mL murine GM-CSF (R&D Systems), for 6 days. The resulting DCs were then magnetically separated by anti-CD11c antibody-coated-magnetic beads (Miltenyi Biotech) and confirmed by anti-CD11c staining. The average separation purity was of, at least, 85%.

3.1.7 Flow cytometry analysis of murine DC subsets

Blood, lymph node and splenic cells were stained with **a**llo**p**hyco**c**yanin (**APC**)-labelled anti-CD11c (HL3), **per**idinin **c**hlorophyll **p**rotein (**PerCP**)-labelled anti-CD8a (53-6.7) or FITC-labelled anti-B220 (RA3-6B2) in combination with anti-CD11b (NS-1), anti-CD86 (GL1) or anti-I-A^b (murine MHC class II molecules) (AF6-120.1) PE-labelled mAbs (BD Biosciences). Samples were analyzed as described above. The DCs were considered CD11c- and I-A^b-positive cells. The cut-off point for positive staining was above the level of the control isotype mAbs.

3.2 Analysis of sialyltransferases (ST) activity

3.2.1 Real-time PCR analysis of ST gene expression

RNA was extracted from 1×10^6 cells using the RNeasy Mini Kit (Qiagen) and GenElute Mammalian Total RNA Purification kit (Sigma Aldrich), and genomic DNA was eliminated from the resulting extract with the RNase-Free DNase Set (Qiagen), as described by the manufacturer. The concentrations of the RNA samples were determined spectrophotometrically. 1 µg of total RNA was reverse transcribed with random primers, using the High Capacity cDNA Archive Kit (Applied Biosystems). PCR was performed in a 7500 Fast system (Applied Biosystems) using TaqManFast Universal PCR Master Mix, primers and Taqman probes provided by Applied Biosystems and following the manufacturer's instructions. The reference sequences detected by each primer/probe set and the Assay ID provided by the manufacturer were the following: ST6Gal-1 (NM173216.1 NM003032.2/Hs00174599_m1); ST6Gal-2 (NM032528/Hs00293264_m1); ST3Gal-1 (NM173344 NM003033/Hs00161688_m1); ST3Gal-2 (NM006927.2/Hs00199480_m1); ST3Gal-3 (NM174969 NM174968 NM006279 NM174972 NM174964 NM174971 NM174963/Hs00196718_m1); ST3Gal-4 (NM006278/Hs00272170_m1); ST3Gal-5 (NM003896.2/Hs00187405_m1); ST3Gal-6 (NM006100.2/Hs00196086_m1); ST6GalNAc-1 (NM018414.2/Hs00300842_m1); ST6GalNAc-2 (NM006456.1/Hs00197670_m1); ST6GalNAc-3 (NM152996.1/Hs00541761_m1); ST6GalNAc-4 (NM175039.1 NM17 5040.1 NM014403.3/Hs00205241_m1); ST6GalNAc-5 (NM030965.1/Hs00229612_m1); ST6GalNAc-6 (NM013443.3/Hs00203739_m1); CD1a (Hs00233332_m1); CD1b (Hs00233507_m1), CD1c (Hs00233509_m1); IL-6 (Hs00174131_m1); IL-12α (Hs00168405_m1); IL-1β (Hs00174097_m1); tumor necrosis factor-α (TNF-α; Hs00174128_m1); IL-10 (Hs00174086_m1); β-actin (4352935E); glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 4333764F). Each reaction was performed in

duplicate. Thermal cycling conditions were 95°C for 20 seconds followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The messenger RNA (mRNA) expression was normalized using the geometric mean of β-actin and GAPDH household genes expression. The relative mRNA levels were expressed in ‰ of the β-actin/GAPDH expression and were calculated by the formula $2^{\Delta Ct}\times1000$, and the relative expression for each gene was calculated by using the formula $2^{\Delta\Delta Ct}$, adapted according to the method described by Livak and Schmittgen (Livak and Schmittgen, 2001). ΔCt stands for the difference between the cycle threshold (Ct) of the amplification curve of the target gene and that of the β-actin/GAPDH. The efficiency of the amplification reaction for each primer-probe was above 95% (as determined by the manufacturer).

3.2.2 Sialyltransferases enzymatic activity assays

Total sialyltransferase (ST) activity was assayed in whole homogenates as previously described by Dall'Olio et al. (Dall'Olio et al., 1996) with minor modifications, using asialotransferrin as N-glycan sialylation acceptor and Galβ1,3GalNAcα1-O-benzyl (Sigma) as O-glycan sialylation acceptor. Cell pellets were homogenized in ice-cold water and the protein concentration assessed by Lowry method (Lowry et al., 1951). The assay mixture contained 50 mM sodium cacodylate buffer, pH 6.2, 0.1%Triton X-100, 4 μM cytidine monophosphate (CMP)-[^{14}C]Neu5Ac, 6 μM CMP-Neu5Ac, one acceptor substrate (8 mg/mL of glycoprotein or 2 mM of Galβ1,3GalNAcα1-O-benzyl) and 50 μg of the protein homogenate, in a final volume of 25 μl. The enzyme reaction was performed at 37°C for 2 h. Controls without exogenous acceptors were run in parallel and their incorporation was subtracted. Mice liver homogenates were used as positive controls.

Two glyco-acceptors were used: glycoprotein and benzyl-glycoside. For the glycoprotein acceptor, the reaction was ended with the addition of 1% phosphotungstic acid in 0.5 M HCl (PTA). Acid insoluble material was collected by centrifugation. The

formed precipitates were washed twice with PTA and once with methanol, boiled in HCl 1 M for 20 min and then transferred to liquid scintillation vials and processed for scintillation counting on a Perkin-Elmer Microbeta® scintillation analyzer (Perkin Elmer). As for the benzyl-glycoside reaction, it was stopped with the addition of 0.5 mL H_2O and products were isolated by hydrophobic chromatography on C-18 SepPak cartridges (Millipore Corp.). After washing with H_2O, the benzyl-glycoside was eluted with acetonitrile and counted by liquid scintillation. The activity was measured as the amount of radioactive sialic acid transferred from the donor to the acceptor substrate per hour and per amount of protein (nmol/h*µg protein).

3.3 Sialidase treatment

Sialic acid removal of moDCs' cell surface was performed by ressuspending moDCs in RPMI 1640 at a cell concentration of 5×10^6 moDCs/mL and incubating them with 200 mU/mL of sialidase from *Clostridium perfringens* (Roche Diagnostics) in serum-free RPMI 1640 for 90 min at 37°C. In some experiments the bacterial surface was desialylated with 135 U/mL of a sialidase from *Arthrobacter ureafaciens* (Sigma Aldrich Ltd), according to the manufacturer's instructions.

In parallel to all sialidase treatments, identical samples were treated in the same conditions with heat-inactivated sialidase (mock treatment). At the end of the incubation, cells were washed and submitted to endocytosis analysis, as described below.

3.4 Endocytosis assay

3.4.1 Endocytosis by moDCs

Endocytosis assays were performed with monocytes obtained by CD14 antibody positive selection, as described above or immature moDCs cultured until 7th day. 3×10^5 cells in RPMI 1640+10% FCS were incubated with 1 mg/mL of either Lucifer yellow CH potassium salt, FITC-dextran (DX, M_r=40,000) and FITC-ovalbumin (Molecular Probes), for 45 min, on ice/4°C or at 37°C. 50 µl of 0.1% Trypan blue solution was added at the end of the incubation to quench the surface attached fluorescence. Cells were then washed twice with PBS+1% FCS and analyzed by flow cytometry, as described.

3.4.2 Endocytosis by BMDC

The endocytosis assays with mouse BMDCs were performed by minor modifications of the above described moDCs endocytosis assay. Briefly, 3×10^5 BMDCs/mL were incubated, for 30 min, with FITC-conjugated ovalbumin at a final concentration of 0.05 mg/mL, at 4°C or 37°C. After stopping the reaction, the cells were stained with PE-labelled anti-MHC II (I-Ab) (AF6-1201) (BD Biosystems). To neutralize cell surface-adsorbed fluorescently labelled ovalbumin, 300 µl 0.1% trypan blue solution was added and cells were washed twice before acquisition by flow cytometry.

3.4.3 Bacterial strain and labelling

For the phagocytosis assays, an *Escherichia coli* K12-derived strain was mainly used in this work. In some experiments, pathogenic *E. coli* isolates from blood cultures and hemocultures obtained from different patients either with urinary infection or septicemia and identified through a Vitek 2 system (Biomerieux) were used. In order to study moDCs and BMDCs phagocytosis, both strains were fluorescently labelled with 0.1 mg/mL of

FITC (Molecular Probes-Invitrogen), at 95° for 1h in 0.1 M sodium carbonate buffer (pH 9.0). Bacteria were then incubated for 1h in PBS, with continuous shaking and stored at -20°.

3.4.4 Phagocytosis assay

Human moDCs/mature moDCs or murine BMDCs (5×10^5 cell/mL) were incubated with 5×10^6 FITC-bacteria, for 1h, at 37°C or 4°C. Incubation time was terminated by adding 0.1% trypan blue solution to quench surface-attached fluorescence. The phagocytosis was analyzed by flow cytometry and confocal microscopy. The internalized bacteria were estimated by flow cytometry, by measuring the mean fluorescence intensity (MFI) of the cells. The MFI values obtained at 4°C (representing basal, background fluorescence) were subtracted from the values obtained at 37ºC. In some experiments, phagocytosis was conducted with human moDCs incubated with 50 μg/mL of either SNA or MAA lectins, or, alternatively, in the presence of 10 μM cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-5-NeuAc) (Sigma Aldrich Ltd.). In the competitive binding assays, 10 mM free synthetic sialic acid (Sigma) was added 30 min before the endocytic agent and left during the assay.

3.5 T cell activation assay

In order to test for T cell activation by moDCs, different parameters of autologous T cell priming assays already described in the literature (Bajana et al., 2007;Verkade et al., 2007) were modified, tested and optimized. Immature human moDCs were generated (as described in the subsection *Human monocyte to moDC differentiation*) from monocytes isolated from PBMCs of healthy, consenting volunteers who had been vaccinated with tetanus toxoid (TT) within the previous 12 months so as to have optimal immunological memory. In these cases, the negative fractions ($CD14^-$ PBMCs) resulting from monocyte isolation were maintained in culture in complete culture medium (previously described) until the mixed lymphocyte cultures with autologous moDCs. On day 5, moDCs were pulsed with or without 5 µg/mL TT (Sigma Aldrich Ltd.) and, 18h later, moDCs were inactivated with 0.05 mg/mL mitomycin C (Sigma Aldrich Ltd.) for 30 min and washed three times with 10 mL RPMI-1640 medium. The $CD14^-$ PBMCs were fluorescently labelled with 5.56 µg/mL **c**arboxy**f**luorescein diacetate **s**uccinimidyl **e**ster (**CFSE**) (Molecular Probes), for 15 min at 37°C, according to the manufacturer's instructions. The labelled cells were then incubated with the autologous, TT-loaded moDCs (treated or mock-treated with sialidase), in the proportion of 8:1 or 4:1 (T cell : moDC ratio), in 96-well round-bottom plates, for 7 days. As a positive control for T cell proliferation, PBMCs were stimulated with 2 µg/mL **p**hyto**h**aem**a**gglutinin (PHA) (Fluka) for 3 days.

Evaluation of the T cell proliferation was performed using flow cytometry, after staining the samples with **a**llo**p**hyco**c**yanin-labelled (**APC**) anti-CD3 (clone 33-2A3) (Immunostep). The T cell activation level was obtained by one of two ways: the number of proliferating T cell, calculated as the percentage of $CD3^+$ cells with relatively decreased CFSE fluorescence intensity vs. day 0 fluorescence intensity; or, alternatively, by determining interferon-γ (IFN-γ) gene expression after 48 hr of co-culture.

3.6 Mouse *in vivo* studies

3.6.1 DC adoptive transfer

The procedure commonly known as 'adoptive transfer' refers to the transfer of exogenously generated cells – in this case, BMDCs – into recipient organisms. Briefly, BMDCs generated and matured as previously described, were gently detached from the Petri dishes after addition of 10-15 mL pre-warmed Accutase (Sigma-Aldrich) at 37°C for 10 min. The detached cells were washed, fluorescently labelled with PKH 67 (Sigma Aldrich) according to manufacturer's instructions, in order to track down end-point migration towards draining lymph nodes, and checked for successful staining by flow cytometry. 1.0×10^7 cells were then ressuspended in 200 µl PBS and subcutaneously injected in the recipient mice hind legs footpads ($5x10^6$ PKH^+ cells / 100 µL in each footpad). After 24h, the recipient mice were euthanized and the draining lymph nodes – namely, popliteal and inguinal lymph nodes – removed. In order to extract the included cells, a similar procedure to the one previously described in the *Lymphoid tissue-resident DCs extraction* subsection. The cells were then stained with fluorescently labelled antibodies for known murine DC markers: PE-anti-CD11b (M1/70), APC-anti-CD11c (N418), APC-anti-B220 (RA3-6B2) and PerCP-anti-I-A^b (M5/114.15.2) (Biolegend).

3.6.2 Inflammatory DC migration

To stimulate DCs to migrate towards draining lymph nodes, 2 mg Alexa Fluor 647-labelled ovalbumin (Invitrogen) were added to 1 mL of an injectable solution of alum (10mg/mL aluminum hydroxide) (Thermo Scientific). 250 µL of this suspension were then subcutaneously injected in the scruff area of WT and ST6Gal-$1^{-/-}$ mice. 48h later, the mice were euthanized and the draining lymph nodes of the neck region, as well as mediastinal lymph nodes, were collected and processed, as described in the *Lymphoid tissue-resident DC extraction* section, in order to extract the therein contained cells. The

collected migratory DCs were, then, fluorescently labelled, as previously described in section 6.1. The migrating, ovalbumin-stimulated DCs were finally detected as, besides being positive for all DCs markers, they also were Alexa Fluor 647-positive.

3.7 Intracellular signalling pathways studies (MAPK western blot)

To assess the activation of intracellular pathways potentially induced by sialic acid removal, moDCs lysates were submitted to SDS-PAGE and western blot assays. Briefly, a fraction of moDCs were cultured, as previously described, and collected. The LPS-stimulated fraction was incubated with 5 µg/mL of LPS (Sigma-Aldrich Ltd.) for 1h, prior to collection. The remainder moDCs were gently harvested and submitted to sialidase treatment, as previously described (section *Sialidase treatment*). In parallel, a mock-treatment with heat-inactivated sialidase was performed. In order to maintain the signalling pathways at the highest possible level, all reactions (sialidase treatment, mock treatment and LPS-stimulation) were stopped by putting the cell suspension on ice. The cells were then centrifuged at 4°C (or lower) and washed with ice-cold PBS, followed by lysis with lysis buffer (50 mM Tris buffer, pH 8.0 + 150 mM sodium chloride) supplemented with a cocktail of protease and phosphatase inhibitors, namely, cOmplete Protease Inhibitor tablets (Roche Applied Science), 1mM sodium orthovanadate (Sigma-Aldrich) and 10 mM sodium fluoride (Sigma-Aldrich). The cells were then mechanically disrupted with the help of a mechanical homogenizer until fully lysed. After protein quantification by the Bradford method, 20 µg of sample protein in loading buffer were loaded per lane of a 12.5% SDS-PAGE gel. The developed gel was then transferred to a PVDF membrane (BioRad) on an ECL TE 77 PWR semi-dry transfer system (GE Life Sciences) for 1h, according to the manufacturer's instructions. The protein-containing membrane was washed in TBS for 5 min and blocked with TBS-Tween 20 (TBS-T) 0.1% (v/v) + 5% BSA for 1 h, at room temperature. The blotting procedure followed by incubating the rabbit anti-human ERK 1/2 and phosphorylated-ERK (pERK) 1/2 antibodies (Cell Signalling Technology, Inc., MA, U.S.A.) were diluted 1:2000 and 1:1000, respectively, in TBS-T 0.1% (v/v) and incubated with the membrane overnight, at 4°C. After washing thrice with TBS-T 0.1% (v/v), the membrane was incubated with a 1:2500 dilution of goat anti-rabbit Fc-horseradish peroxidase (HRP) conjugate antibody

(Sigma-Aldrich) in TBS-T 0.1% (v/v) + 5% BSA (w/v) for 1h, at room temperature, and finally washed as previously mentioned. The membrane was then developed by addition of Immobilon chemoluminescent substrate to the membrane for 5 min and digitally revealed in a ChemiDoc™ XRS+ system (BioRad) with Image Lab™ software, which also allowed the quantification of the blots' intensities and densities. After blotting for the pERK 1/2 protein, the membranes were 'stripped' of antibodies by incubating them with a mild stripping buffer (15 g/l glycine + 1 g/l SDS + 1% (v/v) Tween-20, pH 2.2) twice for 10 min and washing them, first, with PBS (twice) and lastly, with TBS-T 0.1% (v/v) (twice). The stripped membrane was, thus, prepared for the blotting assay with the anti-ERK 1/2 antibody, as above described.

3.8 Microscopy studies

3.8.1 Confocal laser scanning microscopy

Human moDCs were allowed to adhere to concanavalin-A-coated cover glasses and then, paraformaldehyde-fixed and permeabilized with 0.1% Triton-X (Sigma). The cell cytoskeleton was stained with phalloidine-Alexa Fluor 633 (Molecular Probes-Invitrogen). Images were acquired with a TCS SP2 AOBS confocal microscope (Leica Microsystem GmbH) with ×40 oil immersion optics and 488 nm and 633 nm laser lines for FITC and Alexa Fluor 633 excitation, respectively. The obtained micrographs were assembled and analyzed with the Leica Confocal software LCS LITE 2.6 (Leica Microsystems).

3.8.2 Nuclear factor-κB translocation

Human moDCs were adhered to cover-slip glasses, fixed and then permeabilized as previously mentioned, blocked with 3% BSA for 15 min and then stained with 1:100 diluted rabbit anti-nuclear factor-κB (NF-κB) p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1h at room temperature. After washing with a PBS + 0.05% Tween-20 (Sigma Aldrich) solution, goat anti-rabbit 568 (Molecular Probes-Invitrogen) in a 1:400 dilution was used as secondary antibody. The moDCs' nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes-Invitrogen). Fluorescent images were obtained with a DMRA2 fluorescent microscope (Leica Microsystem) and merged with IMAGEJ software (National Institutes of Health). The percentage of cells with translocation of the NF-κB to the nucleus was determined through the analysis of a minimum of 600 cells in each condition.

3.9 Rho GTPases activation assay

Human moDCs were FBS-starved for 24h, to set the basal state of the GTPase activation, and then treated under various conditions. Cell lysates (0.6 mg/mL of total protein) were prepared and the Rac1 and Cdc42 activation was determined using the G-LISA Activation Assay Biochem Kit (Cytoskeleton), according to the manufacturer's instructions. The principle behind the assay is, in brief, a classical 'sandwich' ELISA: a Cdc42- or Rac1-GTP-binding protein is immobilized (from manufacture) on the wells of a 96-well plate. Active, GTP-bound Cdc42 or Rac1 in moDCs cell lysates were recognized and bound by the immobilized protein on the wells. A secondary, Rac1- or Cdc42-specific antibody with horseradish peroxidase conjugate (HRP) conjugate closes the 'sandwich' and allows colorimetric detection after substrate addition. The final reaction was photometrically measured by absorbance at 490 nm. In parallel, positive (Rac1 or Cdc42 protein) and negative (no protein) controls were also assayed. The amounts of activated Rac1 or Cdc42 (arbitrary units) were based on the optical density values obtained after subtracting negative control values.

3.10 Determination of α2,6-sialylated proteins

3.10.1 SNA-affinity protein separation

In order to isolate the α2,6-sialylated proteins, we performed lectin affinity chromatography assays. For that, 5 x 10^8 moDCs were lysed in 5 mL RIPA buffer (Sigma Aldrich Chemical Co.). The SNA-agarose column (EY Labs) column was then equilibrated with PBS, according with the manufacturer's instructions and 3 mL of lysate was applied to the column to bind. The non-bound fraction was eluted with 6 mL PBS and the bound fraction was eluted with 3 mL of elution buffer (0.1 M lactose in PBS). All fractions (non-bound and bound) were collected. After sample elution, the column was extensively washed and regenerated with 20 mL of a 1.2M NaCl solution.

The collected bound fraction was concentrated from a 3 mL suspension volume to a 700 µL volume using an Amicon Ultracel 3K column and the separation efficiency was assessed by SDS-PAGE and western blot.

3.10.2 SDS-PAGE and lectin western blot

The SNA^+ positive fraction was run on a 12.5% SDS-PAGE gel, using 25 µg of protein/lane (as quantified by the Bradford method). The gel was transferred to a PVDF membrane (Bio-Rad) using a semi-Dry transfer unit (General Electric), for 1h, according to the manufacturer's instructions. The membrane was then blocked for 1h, at room temperature, with a 1x PBS + Tween 0.1% (PBS-T) + 1x Carbo-Free (Vector Labs) solution, washed and undergone a 2-step blotting: first, a 1h incubation with SNA-digoxigenin (1:1000 dilution) in PBS-T 0.1% + 10 mg/mL BSA, with double washing; second, a 1h incubation with anti-digoxigenin-HRP (Roche Diagnostics) (1:20000) dilution) in PBS-T 0.3% + 10 mg/mL BSA, with double washing. The blotted membrane

was then incubated with Immobilon chemo-luminescent substrate (Millipore Co.) and as above described (section 3.7).

3.10.3 MALDI TOF/TOF assay

Glycoprotein samples were separated by SDS-PAGE on 12.5% polyacrylamide gels and the bands revealed by Coomassie. The bands of interest were manually excised and digested in-gel with trypsin. Briefly, they were washed three times with 25 mM ammonium bicarbonate/50% acetonitrile (ACN), one time with ACN and dried. Twenty-five µl of 10 µg/mL sequence grade modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate were added to the dried gel pieces and the samples were incubated overnight at 37 °C. Extraction of tryptic peptides was performed by adding 10% formic acid (FA)/50% ACN (3×) being lyophilized in a SpeedVac (Thermo Fisher Scientific). Tryptic peptides were ressuspended in 15 µL of a 0,5% acetonitrile/1% TFA solution.

3.10.4 Protein Identification and Data Mining

Protein identification was performed as previously described by Vitorino et al (Vitorino et al., 2010). Tryptic digests were separated with a C18 Pepmap (Dionex) column on an Ultimate 3000 (Dionex/LC Packings) nano-HPLC and fractions were collected with a Probot (Dionex/LC Packings) directly onto a matrix-assisted laser desorption ionization (MALDI) plate. The MALDI-TOF/TOF (time-of-flight) mass spectrometry (MS) analysis was performed on a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems). The MS spectra acquired were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems). Protein identification was achieved with a search performed against the Swiss-Prot protein database for *Homo sapiens*. *N*-Glycosylation in human proteins was predicted by use of the NetNglyc 1.0 server

(http://www.cbs.dtu.dk/services/NetNGlyc), an artificial neural network that examines the sequence context of Asn-Xaa-Ser/Thr (where Xaa is not Pro) sequences. *O*-Glycosylation was predicted by use of the NetOglyc 3.1 server (http://www.cbs.dtu.dk/services/NetOGlyc) that produces neural network predictions of mucin-type GalNAc *O*-glycosylation sites in mammalian proteins (Julenius et al., 2005).

3.11 Statistical analysis

When necessary, data were analyzed for statistical significance using the Student's t-test and ANOVA One-way at 95% confidence level, as appropriate, using GraphPad Prism versions 4.0, 5.0 and 6.0 (GraphPad Software, Inc., La Jolla, CA, U.S.A). Correlations between variables were tested using the Spearman correlation analysis. All data were expressed as means of standard errors of independent assays, i.e. using cells from different donors. *p* values inferior to 0.05 were considered as significant.

Section 4

RESULTS AND DISCUSSION

4.1 Sialylation profiling of DCs

4.1.1 Characterization of sialylation in DCs

There are increasing evidences that sialic acid plays a role in the innate and adaptive immune response. For instance, T cells depend on O-glycan α2,3-sialylation for migration to the periphery and mice from ST6Gal-1$^{-/-}$ mice present impaired humoral response (Hennet et al., 1998;Priatel et al., 2000a). Sialic acid usually terminates glycosidic structures and is, for that reason, a likely candidate to participate in the modulation of several biologic processes (Varki, 2008). As such, **we hypothesized that terminal sialic acid influences the main DC immune functions**.

To understand this, we first analyzed the profile of cell surface sialylation during the *in vitro* moDC generation from monocytes. For this purpose, we have first examined the surface sialylation pattern in monocytes and respective derived immature DCs, at 2 days-interval during differentiation, using the following specific glycan-recognizing proteins:

- the ***S**ambuccus **n**igra* lectin (or **a**gglutinin) (**SNA**) – which recognizes mainly sialic acid α2,6-linked to lactosamine (6′ sialyl-Galβ1,4GlcNAc);

- the ***M**aackia **a**murensis* lectin(or **a**gglutinin**)** (**MAA**) lectin recognizes mainly sialic acid α2,3-linked to lactosamine (3′ sialyl Galβ1,4GlcNAc) or, to a lesser extent, linked to Galβ1,3GalNAc;

- the *Arachis hypogaea* lectin (**PNA**, from '**p**ea**n**ut' **a**gglutinin), a lectin specific for the Core 1 structure of the O-linked chains (Galβ1,3-GalNAc, also known as T antigen).

SNA binding studies showed that monocyte surface had a low binding density of such a structure, which increased during differentiation into moDC, reaching a maximum at day 6 of differentiation (Fig. 4.1), suggesting a higher α2,6-sialic content in moDCs N-glycans. MAA binding increased dramatically during the beginning of the moDC differentiation (maximum at day 2), decreasing slightly until moDCs were fully differentiated (Fig. 4.1), suggesting that monocytes present a low density of α2,3-linked

sialic acid, when compared with moDC. On the other hand, the overall reactivity of PNA was much lower than shown by previous lectins and it increased slightly during the differentiation process (Fig. 4.1). PNA binding could be hindered by sialylation of the galactose or of the GalNAc residues in the T antigen.

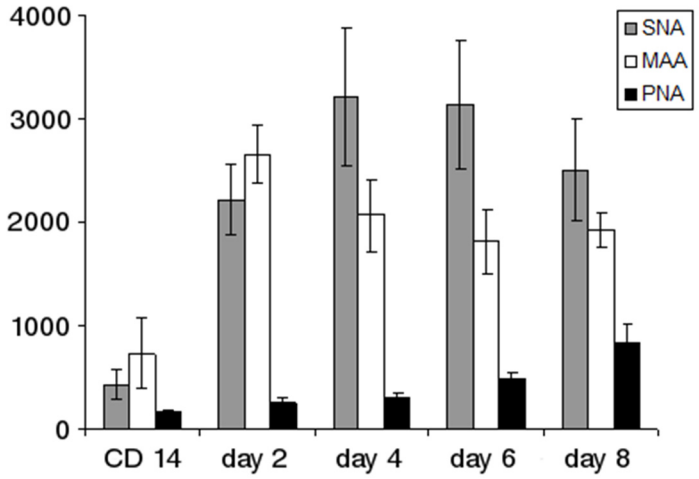


Fig. 4.1 - Lectin binding to the cell surface of monocytes (CD14) along its differentiation into DCs. Results are the mean fluorescence intensity (MFI) obtained by flow cytometry of at least four independent assays. SNA lectin results are represented in grey, MAA in white and PNA in black.

4.1.1.1 Sialyltransferase expression alterations during moDC differentiation

To investigate the molecular bases of the sialylation changes accompanying the moDC differentiation, we studied the mRNA expression of relevant sialyltransferases (STs). When a marked upregulation of the ST gene expression was observed, we also analyzed the enzyme activity level, using specific substrates. For the gene expression two parameters were considered: the relative mRNA levels (Fig. 4.2A, 4.2C) and, for differentiation experiments, the expression relative to the monocytes' expression (Fig. 4.2B, 4.2D).

4.1.1.1.1 STs involved in α2,6-sialylation of N-linked chains

The most relevant enzyme involved in α2,6-sialylation of lactosamine, giving rise to 6' sialyllactosamine (6′ sialylGalβ1,4GlcNAc) is by far ST6Gal-1. Although a second ST6Gal has been cloned (Takashima et al., 2002;Krzewinski-Recchi et al., 2003), we didn't find any level of expression in moDCs and ST6Gal-2 was not further considered.

As revealed by real time PCR analysis, the level of ST6Gal-1 mRNA expression increased during moDC differentiation ($p < 0.01$), closely paralleling the increase of SNA reactivity (Fig. 4.2C and D). Nevertheless, the major correlation is found between the ST6Gal-1 expression and the ST activity (r=0.98, p=0.017), measured with the ST6Gal-1-specific acceptor, asialotransferrin (Dall'Olio et al., 2006) (Fig. 4.2E). Altogether, these results indicate that moDC differentiation is characterized by a marked increase of ST6Gal-1 gene expression, thus resulting in increased enzyme activity and increased α2,6-sialylation of cell surface lactosaminic termini.

4.1.1.1.2 STs involved in α2,3 sialylation of N-linked chains

The α2,3-sialylated lactosaminic chains can be sialylated by, at least, three different STs – namely ST3Gal-3, -4 and -6. MAA staining of DCs was positive, showing that these structures are expressed by DCs, with their expression increasing during moDC differentiation.

The pattern of ST3Gal-4 expression by moDCs (Fig. 4.2B) resembles the changes of MAA reactivity, although this correlation is not statistically significant (p=0.360) and the ST3Gal-4 mRNA appears to be expressed at a low level (Fig. 4.2A). On the other hand, the expression of ST3Gal-3 and -6 mRNA is higher, but shows little changes during differentiation. This finding suggests that ST3Gal-4 contributes to the α2,3-sialylation of lactosaminic chains in differentiating monocytes, but the contribution of ST3Gal-3 and -6 remains open.

4.1.1.1.3 STs involved in α2,3-sialylation of O-linked chains

The α2,3-sialylation of galactose residues present in core 1 O-glycans is mediated by ST3Gal-1, -2 and -4 sialyltransferases. According to the real time PCR analysis, besides the above mentioned ST3Gal-4 upregulation, both ST3Gal-1 and -2 genes are also upregulated during moDC differentiation with ST3Gal-1 showing a significant increased expression ($p<0.05$) (Fig. 4.2B). To corroborate the increased expression of the corresponding enzymes, we have tested the ST activity using Galβ1,3GalNAc-O-benzyl as an acceptor. The ST activity towards the core 1 structures increases during the moDC differentiation process (Fig. 4.2E). These observations could, in some way, justify the observed low reactivity of PNA, but not its slight increase. It is possible that, at that stage, cells are already saturated with sialylated Galβ1,3GalNAc O-glycans (sialyl-T antigens) and, in this context, any increase in α2,3-ST expression will have a negligible influence. In fact, we have observed that moDCs dramatically increased their reactivity with PNA lectin after sialidase treatment (Fig. 4.2F), evidencing a significant content of O-linked Galβ1,3GalNAc masked by sialic acid in these cells.

4.1.1.1.4 ST involved in α2,6-sialylation of O-linked chains.

As shown in Fig. 4.2C, amongst the genes coding for STs able to catalyze the α2,6-sialylation of the GalNAc residues present on O-glycans (ST6GalNAc-1, -2, -3 and -4), ST6GalNAc-4 is the highest expressed, followed by ST6GalNAc-2 and 4. ST6GalNAc-1 relative mRNA level was very low (<0.01‰ of β-actin/GAPDH) and was not considered further (Fig. 4.2C). ST6GalNAc-3 was fairly expressed in monocytes, but its level decreased during moDC differentiation. Given the ST6GalNAc-4 high mRNA expression (Fig. 4.2D) and its restricted substrate specificity, recognizing only sialylated Galβ1,3GalNAc *O*-glycans (sialyl T antigen) (Harduin-Lepers et al., 2000), it is likely that disialyl-T antigens are expressed by monocytes and moDC.

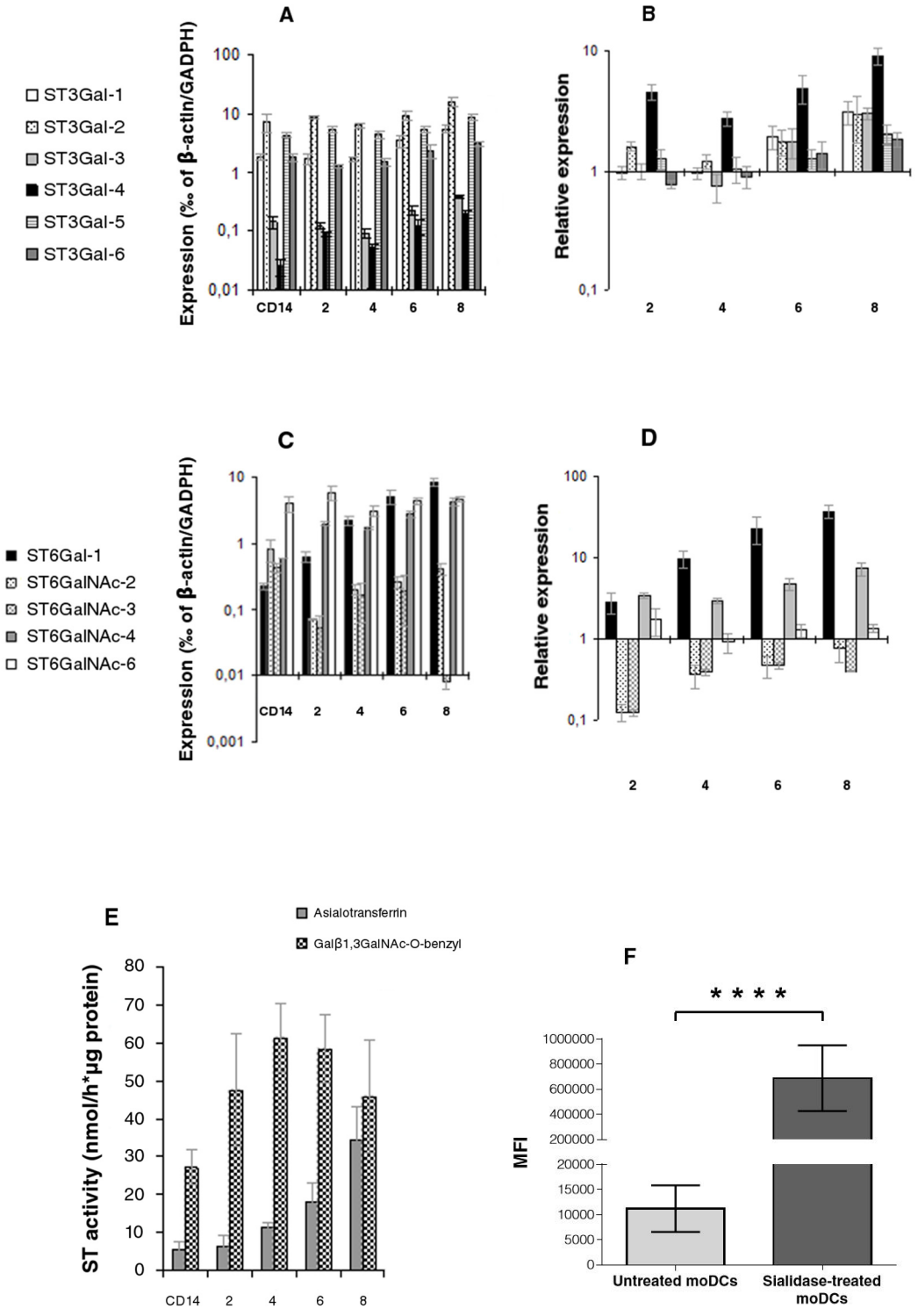


Fig. 5.2 – Alterations in sialylation profile during differentiation of monocytes into moDCs. (**A**, **B**, **C** and **D**) Relative mRNA level and relative expression of α2,3-sialyltransferases (A, B) and α2,6-sialyltransferases (C, D) genes. The mRNA expression level of each gene relative to the endogenous controls, β-actin and GAPDH, expression (A, C) was measured from samples collected each 2 days during the differentiation of monocytes (CD14) into DCs (day 2, 4, 6 and 8) and during DC maturation with IFN-γ , IL-1β, TNF-α and LPS. The relative expression compares the expression of a given gene at

day 2, 4, 6 and 8 relative to the respective expression in monocytes and upon DC activation with IFN-γ, IL-1β, TNF-α and LPS, relative to control, immature DCs at day 8 (B, D). The results are the mean of 4 independent assays. *Data from ST3GalNAc-3 expression at minimum and omitted from the graphs;

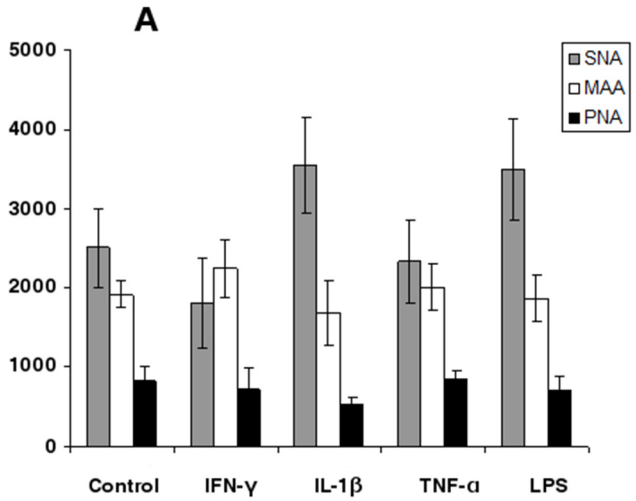
(**E**)- Level of sialyltransferase activity using asialotransferrin and Galβ1,3GalNAcα1-O-Benzyl glycoside as acceptor substrates. The activity was assessed in sample homogenates collected at two days intervals during the differentiation of monocytes (CD14, control) into DCs (day 2, 4, 6 and 8). The results represent the mean of, at least, 4 independent assays. Mice liver homogenates (M) were used as assay control;

(**F**) PNA lectin staining of moDCs prior and after sialidase treatment, as assessed by flow cytometry. The results represent the mean of 11 independent assays. **** $p < 0.0001$

4.1.1.2 Maturation induces changes in the sialylation profile of moDC

To test the influence of maturation in the sialylation profile, we separately stimulated moDC with cytokines that act as diverse pro-inflammatory mediators, namely, TNF-α, IL-1β, LPS or IFN-γ.

LPS and IL-1β stimuli lead to an increased SNA reactivity, contrarily to IFN-γ and TNF-α, which promote a lower SNA reactivity, suggesting the additional expression of α2,6-sialylated lactosamines in the former (Fig. 4.3A). Nevertheless, the ST6Gal-1 gene expression (Fig. 4.3B) and ST activity towards asialotransferrin (Fig. 4.3F) decrease upon all the stimuli and both parameters are highly correlated (r=0.98, p=0.017). MAA reactivity is not significantly affected upon maturation (Fig. 4.3A), although the expression of ST3Gal-2 and -4, which are involved in the synthesis of MAA ligands, decreases with maturation (Fig. 4.3C). This apparent inconsistency can be explained considering that the glycosyltransferases-product relationship is not necessarily linear. In fact, the level of an oligosaccharide product can be regulated at multiple levels, including the action of glycosyltransferases competing for the same acceptor and the action of glycosidases. On the other hand, the ST3Gal-1 expression increases with all maturation stimuli, except TNF-α (Fig. 4.3C), and it is significantly correlated with ST activity for Galβ1,3GalNAc-O-benzyl acceptor (r=0.97, p=0.040). Accordingly, following IL-1β, LPS or IFN-γ stimulation, the PNA reactivity decreases slightly (Fig. 4.3A), suggesting moDC carry out *de novo* synthesis of sialylated T antigens.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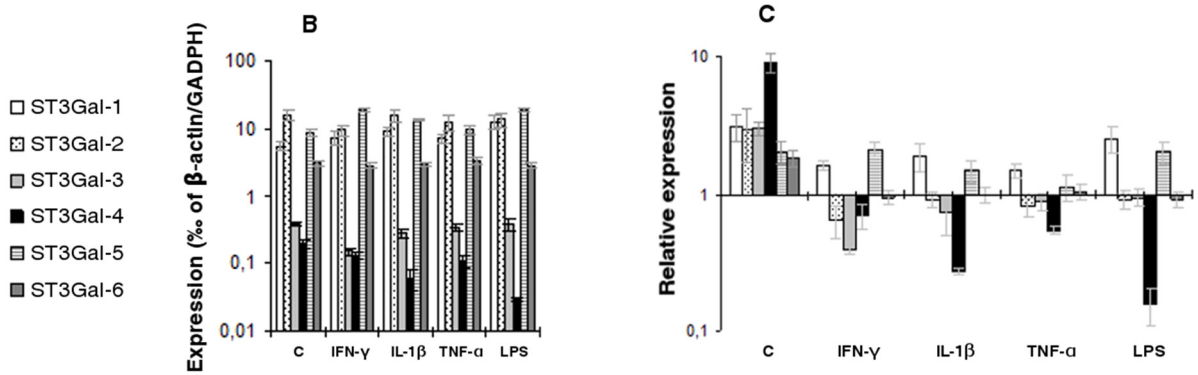
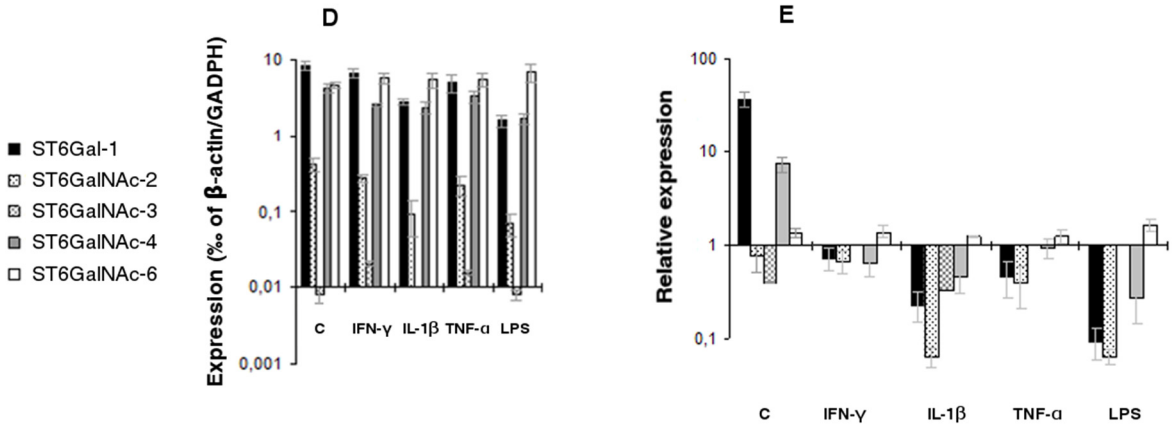
4000
3000
2000
1000
0
SNA
MAA
PNA
Control
IFN-γ
IL-1β
TNF-α
LPS


ST3Gal-1
ST3Gal-2
ST3Gal-3
ST3Gal-4
ST3Gal-5
ST3Gal-6
B
Expression (‰ of β-actin/GADPH)
100
10
1
0,1
0,01
C
IFN-γ
IL-1β
TNF-α
LPS
C
Relative expression
10
1
0,1
C
IFN-γ
IL-1β
TNF-α
LPS


ST6Gal-1
ST6GalNAc-2
ST6GalNAc-3
ST6GalNAc-4
ST6GalNAc-6
D
Expression (‰ of β-actin/GADPH)
10
1
0,1
0,01
0,001
C
IFN-γ
IL-1β
TNF-α
LPS
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Relative expression
100
10
1
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C
IFN-γ
IL-1β
TNF-α
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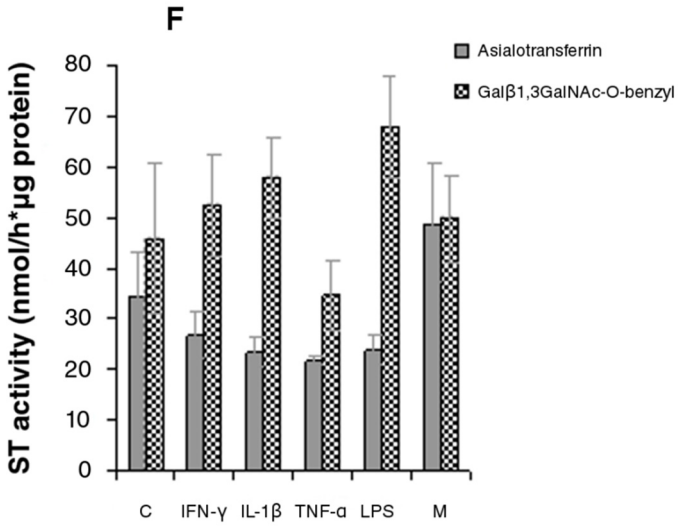


Fig. 4.3 – Alterations in sialylation profile during maturation of moDCs (**A**) Lectin binding to cell surface of immature moDCs (Control) and DC maturated with IFN-γ, IL-1β, TNF-α and LPS. Results are the mean fluorescence intensity (MFI) obtained by flow cytometry of at least four independent assays. SNA lectin results are represented in grey, MAA in white and PNA in black.

(**B**, **C**, **D** and **E**) Relative mRNA level and relative expression of α2,3-sialyltransferases (B and C) and α2,6-sialyltransferases (C and D) genes. The mRNA expression level of each gene relative to the endogenous controls, β-actin and GAPDH, expression (B and D) was measured from samples collected during DC maturation with IFN-γ, IL-1β, TNF-α and LPS. The relative expression compares the expression of a given gene upon DC activation with IFN-γ, IL-1β, TNF-α and LPS, relative to control, immature DCs at day 8 (C and E). The results are the mean of, at least, 4 independent assays. *Data from ST3GalNAc-3 expression at minimum and omitted from the graphs.

(**F**)- Level of sialyltransferase activity, using asialotransferrin and Galβ1,3GalNAcα1-O-Benzyl glycoside as acceptor substrates. The activity was assessed in sample homogenates collected during DC maturation with IFN-γ, IL-1β, TNF-α and LPS, as described in the *Materials and Methods* section. As control (C) condition, fully differentiated, immature moDCs were used. The results represent the mean of, at least, 4 independent assays. Mice liver homogenates (M) were used as assay control.

4.1.1.3 Discussion

The importance of sialic acid in the innate and adaptive immune responses is becoming increasingly evident. It is known that the surface α2,6- and α2,3-sialylation of B and T cells is modulated according to the cell differentiation and maturation (Moody et al., 2003;Marino et al., 2004). DCs are essential to orchestrate the immune response mediated by lymphocytes. However, many DC features, such as the sialylation role, are still poorly understood.

In this subsection, we showed that **DCs express significant amounts of sialylated structures during differentiation from monocyte precursors and that said expression correlates with the expression level of specific sialyltransferases during differentiation**.

The analysis of the ST gene expression demonstrated that **all α2,3- and almost all α2,6-ST genes are expressed**. Clearly, the α2,3- and α2,6-sialylation profile of monocytes and moDCs is a commitment of several STs encoded by different genes.

We have observed an increased expression of α2,3-sialylated O-glycans and α2,6- and α2,3-sialylated N-glycans during the differentiation of monocytes into moDCs. It is known that during moDC generation the cell suffers profound mechanism changes, including the acquisition of specific features such as increased capacity of antigen uptake and the expression of antigen presenting molecules (MHC class I, MHC class II and CD1 molecules), adhesion and co-stimulatory molecules (reviewed in (Banchereau and Steinman, 1998a)). It is, therefore, likely the observed sialylation changes are related with the mechanisms changing during moDC.

The expression of sialylated molecules in some cases appears to be under the control of a single ST, whereas in other cases their biosynthesis appears to be more complex. For example, the increase of α2,6-sialylated lactosamines we detected using SNA lectin during the moDC differentiation clearly relates with the increased sialyltransferase activity on asialotransferrin and the increase of ST6Gal-1 transcript. ST6Gal-1 is highly expressed in B cells and one of its best known products is the counter receptor of CD22 (Collins et al., 2002) but its specific substrates in DCs are still unidentified. It is possible that, similarly to B cells, this 6′ sialyl-lactosamines are ligands for inhibitory receptors on the surface of DCs or effector T cells, during the antigen presentation.

The increased reactivity with MAA lectin, we observed during moDC differentiation, could be attributed to ST3Gal-3, ST3Gal-4 and/or ST3Gal-6. The transcripts of all these three enzymes show a tendency toward upregulation during differentiation, but ST3Gal-

4 high expression at the beginning of moDC differentiation resembles more the level of MAA reactivity. It is reasonable to propose that, although ST3Gal-4 may have a higher contribution, **all three enzymes contribute to the biosynthesis of MAA ligands in DCs**. The low PNA lectin reactivity together with Galβ1,3GalNAc-O-benzyl acceptor enzymatic assays indicate that differentiating moDCs express the Core 1 structure of the O-linked chains (T antigen) masked by sialic acid. The T antigen can be sialylated in α2,3-linkage to Gal by at least three different sialyltransferases, namely ST3Gal-1, -2 and -4, yielding the sialyl-T antigen which, in turns, can be further sialylated by ST6GalNAc-1, -2, -3 or -4, generating the disialyl T-antigen (reviewed in (Dall'Olio and Chiricolo, 2001)). According to the real time PCR analysis, all the genes coding for enzymes involved in the α2,3-sialylation of T antigen undergo upregulation during moDC differentiation, suggesting the expression of this structure. During moDC generation, the ST3Gal-1 expression variations are more similar to the ST activity towards Galβ1,3GalNAc-O-benzyl acceptor. This enzyme is known to exhibit a higher acceptor substrate selectivity towards O-linked oligosaccharides of glycoproteins (Kitagawa and Paulson, 1994) than ST3Gal-2 and ST3Gal-4, accepting preferentially glycolipids and N-glycans, respectively (Kitagawa and Paulson, 1994;Kim et al., 1996). Therefore, **it is likely that the ST3Gal-1 gene is the major responsible for the α2,3-sialylation of T antigens in moDC**. In addition, the increased expression of ST3Gal-1 during the stimulation of moDCs is significantly correlated with the ST activity over the T antigen, indicating an enhanced content of these sialylated structures. Nevertheless, masking by sialylation does not appear to be the major mechanism of regulation of T antigen expression in differentiating moDC, as the PNA reactivity does not decrease (as one could expect) but, rather, slightly increases. This means that, in moDCs, the main regulation of the T antigen probably occurs at the level of peptide:GalNAc transferases and/or of β1,3-galactosyltransferase (Brockhausen, 1999).

The STs expressed in differentiating moDC, involved in the α2,6-sialylation of the GalNAc residue of the sialyl-T structure, ST6GalNAc-2, -3 and -4, show opposite behaviors, being the first two downregulated and poorly expressed, and the latter slightly upregulated during differentiation. Hence, it is expected that **the moDC differentiation process poorly affects the expression of the disialyl-T antigen**.

The biosynthesis of sialyl-Le^x, the functional motif of selectin ligands, requires the α2,3-sialylation of lactosamine, followed by the α1,3-fucosylation of 3′ sialyl-lactosamine. The sialylation reaction may be mediated by ST3Gal-4, -6 and, to a lesser degree, ST3Gal-3 (Okajima et al., 1999). The expression of selectin ligands has been demonstrated in DCs derived from bone marrow (Kieffer et al., 2001) and we have also observed sialyl-Le^x antigens in moDCs (Silva et al., 2011). In neutrophils, ST3Gal-4 was found to play the major role (Ellies et al., 2002), although, in this work, ST3Gal-4 exhibited a relative low mRNA level in monocytes and moDCs (Fig. 4.2a), whereas ST3Gal-6 is probably the main ST involved in the biosynthesis of sialyl Le^x in these cells. Nevertheless, further studies are necessary to investigate this assumption.

We have analyzed the influence of maturation stimuli in the sialylation pattern of moDC. We used several inflammatory stimuli, namely TNF-α, IL-1β, LPS and IFN-γ, known for activating DC to polarize T cells towards a T_h1 profile, yet with only known slight phenotypical or functional differences (Ardavin et al., 2001). According with the lectin reaction patterns, **the major moDC phenotype difference after maturation was found in the expression of α2,6-sialylated lactosamines**, with each stimulus leading to distinct expression of these structures. Regarding the ST gene expression, all these stimuli lead to a downregulation of ST6Gal-1 and ST3Gal-4 while upregulating ST3Gal-1. Another research group has recently reported that tolerogenic, immature DCs have a high α2,6-linked sialic acid density, which decreases after stimulation with a cytokine cocktail (IL-6, IL-1β, TNF-α and PGE2) (Jenner et al., 2006). Concomitant with our

results, these authors have also found that ST6Gal-1 is downregulated after stimulation, indicating that this is probably a common pattern of the moDC maturation. It is known that the DC maturation leads, generally to the downregulation of the antigen-uptake machinery, upregulation of adhesion and co-stimulatory molecules and peptide-MHC complexes density on DC cell surface and ultimately the polarization of different T cell subsets (Banchereau and Steinman, 1998b). Based on these facts, **it can be assumed that the observed expression changes of ST3Gal-1, -4 and ST6Gal-1 may be associated with the expression or regulation of specific molecules involved in the maturation**.

4.1.2 DC subtypes and tissue distribution in ST3Gal-1- and ST6Gal-1-deficient mice

Given our previous observations relating DC differentiation and sialylation, namely, the modification of sialylation during monocyte to moDC differentiation and maturation, we sought to find its functional impact and, ultimately, why these alterations occurred.

DCs are generated in bone marrow from a common myeloid progenitor and, in later stages of differentiation, they migrate to different peripheral tissues where they perform their functions (Shortman and Naik, 2007). Given the known influence of glycosylation/sialylation in DC migration (Le Marer and Skacel, 1999;Yakubenia et al., 2008), we hypothesized that **sialylation alterations may, thus, translate into alterations in DC function**, and that **one of the first observable alterations *in vivo* would be alterations in DC subtype's tissue distribution**. The chosen DC subset markers were, at the time of this work, the accepted standards for DC subset analysis. We first tested the distribution of lymphoid ($CD8\alpha^+$), plasmacytoid ($CD45R/B220^+$) and conventional ($CD11b^+$) within mouse DC ($CD11c^+$ $I\text{-}A^{b+}$) subsets in blood, spleen and

lymph nodes of ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ mice. Interestingly, we found differences in the ST3Gal-1$^{-/-}$ mice, which presented not only an increased percentage of total DCs, but also a significant inferior percentage of CD8α^{+} DCs and, conversely, increased number of B220^{+} DCs in the three analyzed sources (Table 4.1). With the exception of B220^{+} DCs in the spleen of ST6Gal-1$^{-/-}$ mice, DC from these mice didn't show any significant alteration in tissue distribution and subset quantity, compared with wild type mice.

Table 4.1. Distribution of dendritic cell subsets in ST3Gal-1$^{-/-}$ and ST6Gal.-1$^{-/-}$ mice compared with wild-type control mice. The values of statistical significance were obtained by comparison of the knocked-out mouse strains with the wild-type mice in the same tissue. DCs, dendritic cells; WT, wild-type; DCs were considered CD11c and MHC II positive cells. N indicates the number of mice used for each determination. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

Tissue		N	% DCs[1]	% Lymphoid DCs (CD8α^{+})[2]	% Plasmacytoid DCs (CD45/B220^{+})[2]	% Conventional DCs (CD11b^{+})[2]
Blood	WT	8	0.938 ± 0.08	4.400 ± 0.88	23.94 ± 2.16	7.353 ± 0.79
	ST3Gal-1$^{-/-}$	6	1.208 ± 0.09 *p = 0.044	0.7183 ± 0.32 ** p = 0.002	43.03 ± 3.62 ** p = 0.002	8.500 ± 2.26
	ST6Gal-1$^{-/-}$	10	0.797 ± 0.06	4.435 ± 0.51	17.98 ± 1.20	5.535 ± 0.69
Spleen	WT	6	6.426 ± 0.89	11.12 ± 1.09	25.19 ± 2.13	25.65 ± 4.94
	ST3Gal-1$^{-/-}$	7	11.82 ± 1.04 ** p = 0.004	7.300 ± 1.30 * p = 0.048	40.56 ± 5.56 * p = 0.048	36.44 ± 2.28
	ST6Gal-1$^{-/-}$	11	5.074 ± 0.72	8.071 ± 0.89	32.10 ± 1.52 * p = 0.025	30.13 ± 3.66
Lymph nodes	WT	7	3.042 ± 0.47	10.19 ± 1.61	35.79 ± 3.45	28.18 ± 6.82
	ST3Gal-1$^{-/-}$	6	7.220 ± 0.44 *** p = 0.0002	3.720 ± 1.81 * p = 0.032	54.80 ± 5.10 * p = 0.015	42.09 ± 6.65
	ST6Gal-1$^{-/-}$	9	3.690 ± 0.75	9.929 ± 1.98	41.11 ± 1.97	34.16 ± 6.24

[1] Percentage related to total leucocytes; [2] Percentage related to total DCs.

4.1.2.1 Discussion

Lymphocytes, granulocytes and neutrophils from ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ mice have been the object of several studies (Hennet et al., 1998;Priatel et al., 2000a;Nasirikenari et al., 2006;Nasirikenari et al., 2010) but, as far as we know this is the first reported characterization of their DCs. By studying the distribution of lymphoid,

plasmacytoid and conventional DC subsets in blood, lymph nodes and spleen, based on the expression of specific markers, we have found significant differences in DC population. **ST3Gal-1$^{-/-}$ mice presented a significantly lower percentage of CD8α^{+} DCs. In contrast, both ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ show increased number of B220^{+} DCs in the periphery** (Table 4.1).

The results here presented corroborate our hypothesis that sialic acid content may influence DC tissue distribution. Deficiencies in ST3Gal-1 and ST6Gal-1 result in significant altered DC subtype populations, underlying the relevance of these sialyltransferases in the distribution of specific subsets. The underlying causes of the observed alterations can occur in two sequential phases of DC lifecycle: differentiation and migration (Lund-Johansen and Terstappen, 1993;Le Marer et al., 1997;Le Marer and Skacel, 1999;Alvarez et al., 2008;Winkler et al., 2012).

Our results show a more relevant role for the ST3Gal-1, which may be justified by its role in the synthesis of a prominent selectin ligand - sialyl-Lewis x (sLe^{x}). Among the three selectins, E-selectin has been described to be a key player both in hematopoiesis and migration, by regulating the stem cell balance between quiescence to differentiation (Winkler et al., 2012) and the stem cell migration from the BM to the blood and from here to the peripheral tissues, especially to the lymph nodes (Alvarez et al., 2008), which could explain the differences found in DC populations. In addition, it is relevant to note that ST3Gal-1$^{-/-}$ CD8^{+} lymphocytes have been described as having a similar CD8/B220 disproportion (as assessed by the Consortium for Functional Glycomics - http://www.functionalglycomics.org/glycomics/ImmunologySummaryServlet?pageType=view&sideMenu=no&summaryId=imm_summary_4_06202003) with a significant reduction of the CD8^{+} T cell population and increase of B220^{+} population, due to defects in T cells maturation (Priatel et al., 2000a). The observed alterations in different DC (CD8+ and B220+) population numbers suggests a higher content of plasmacytoid (B220^{+}) DCs. From the therapeutic point of view alterations in DC subsets may prove

useful in several dysfunctional immune disorders. However, further analysis are required to validate the relationship of ST3Gal-1 expression and plasmacytoid DC abundance.

As for ST6Gal-1, regarding the observed $B220^{+}$ increase in spleen and lymph node, it has been described as influencing the hematopoietic cell precursors detachment from the bone marrow during DC differentiation (Le Marer and Skacel, 1999). In line with the available literature, the absence of ST6Gal-1 may, thus, also inhibit DC precursor detachment from the BM matrix, lowering circulating DC levels and hampering blood to peripheral tissue DC transition. This, for the same adhesion properties and over time, would lead to DC accumulation in the spleen and other SLOs.

In this context, our results contribute to existing evidence, **further enlightening the role of ST3Gal-1 (more relevant) in these stages of differentiation, specifically as a regulator of DC population homeostasis and tissue distribution**. Using the KO model, the absence of these STs in our assays is similar to the downregulation of these STs upon maturation, but taken to an extreme. Since, as we've presented in section 4.1.1, after inflammation induction we observe a downregulation of STs, the KO model could, biologically, could be organically interpreted as permanent state of inflammation, inducing the release of a higher number of DCs to migrate towards an insult site. This may be of particular relevance regarding exogenously induced hematopoietic stem cell ablation, as during chemotherapy, where immune cell repopulation is required.

4.1.3 Sialylation-induced DC maturation

DC maturation is a natural process arising from the encounter of DC with extracellular stimuli (antigenic or not), for instance, during endocytosis/phagocytosis. Curiously, **both endocytosis and maturation seem to be co-dependent, since an increase of maturation is accompanied by a decrease in endocytic activity in moDCs**. Knowing the overwhelming majority of the stimuli interacts with DCs via cell surface receptors, **we**

questioned if a change in the sialylation content of DCs would alter their maturation status, thus impacting their endocytosis performance. To confirm this suppositions, we also analyzed the maturation levels of sialidase-treated and non-treated moDCs, submitted to endocytosis or not, via the expression of maturation markers, namely, MHC class II, CD80 and CD86. We also tested moDCs which had not undergone endocytosis (assays performed at 4°C in the presence of ovalbumin) or were not treated (assays performed at 37ºC in the presence of "mock" (heat-inactivated) sialidase) (Fig. 4.4). DC maturation was unlikely to be the result of heat-stable factors in the sialidase preparation, such as bacterial endotoxins, because **the control DCs treated with heat-inactivated sialidase did not show decreased endocytosis**. According to our results, the expression level of these markers was significantly elevated in the cells treated with sialidase, although, except for MHC I, with less extent (Fig. 4.4A). The sialidase treatment alone (4°C condition) caused a significant increase of MHC I and MHC II expression in moDCs, as well as the expression of co-stimulatory molecules, CD80 and CD86 (Fig. 4.4B) – a change even more pronounced when followed by endocytosis (37°C condition). We also observed the same cumulative effect of the sialidase treatment and phagocytosis on moDCs maturation, as represented in Fig. 4.4C, translated in higher expression of MHC II expression. Besides MHC molecules, human moDCs prominently express other antigen-presenting molecules, the CD1 family (CD1a, -b and -c) that mediate the presentation of lipid and glycolipid antigens to T cells (Porcelli et al., 1998). However, the trafficking of such molecules to the plasma membrane is generally independent of the DC maturation stage (van der Wel et al., 2003), except in the case of strict stimulus (Martino et al., 2006;Rigano et al., 2007), so we haven't addressed this maturation feature.

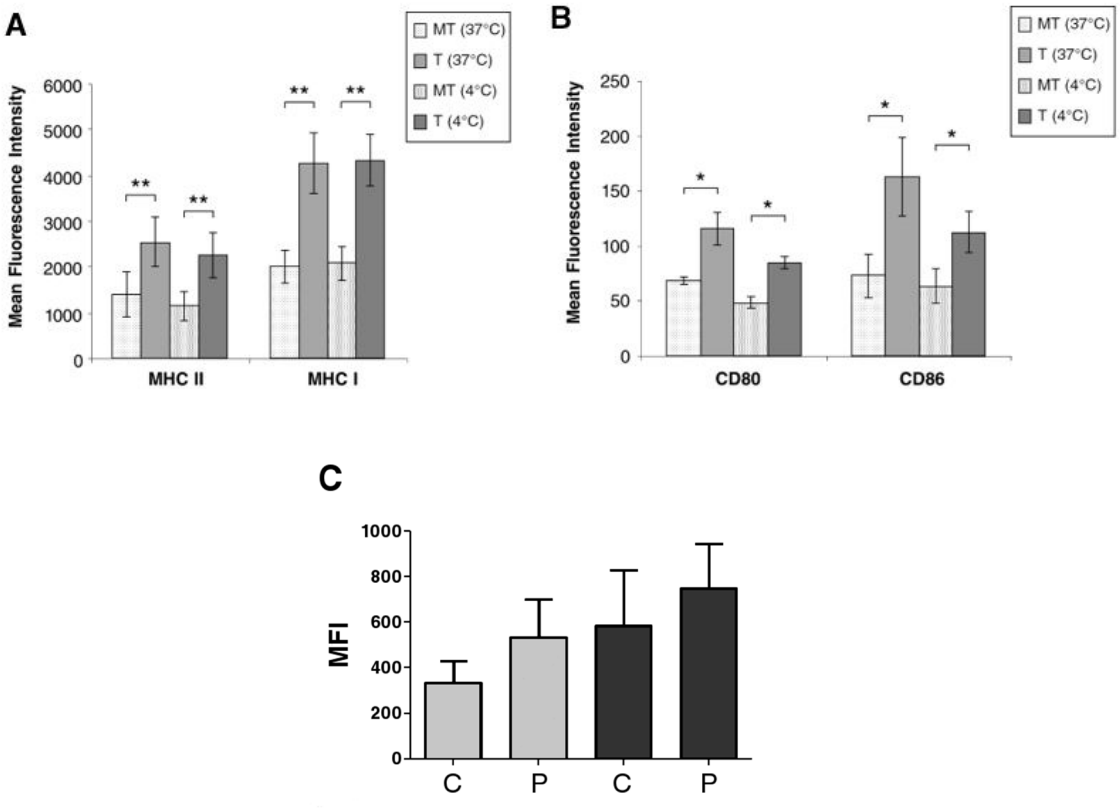


Fig. 4.4 – Maturation levels in sialidase-treated and mock-treated human moDCs, following endocytosis and phagocytosis. (A and B) Sialidase treatment increases expression of major histocompatibility complex class II (MHC II) and class I (MHC I) (A), CD80 and CD86 (B) in human moDCs after sialidase treatment and endocytosis. Sialidase treated (T) or mock-treated (i.e. inactivated sialidase) (MT) moDCs were pulsed with the endocytic agent ovalbumin and incubated for 1h at 37°C or 4°C (control). After the endocytosis assay, the expression of MHC II (HLA DR), MHC I (HLA ABC) and the co-stimulatory molecules CD80 and CD86 were evaluated by flow cytometry. The results are the mean of the mean fluorescence intensity (MFI) values of at least 3 individual experiments ± SEM. Significantly different values (* $p<0.05$ or ** $p<0.01$ paired Student's t-test) were observed for all the maturation markers employed, comparing sialidase-treated with mock-treated human moDCs, either in cells that had internalized ovalbumin (assay at 37°C) or in the control cells (assay at 4°C). (C) moDCs were treated with sialidase or left untreated, and then incubated or not (control) with *E. coli***.** The expression of MHC was evaluated by flow cytometry after staining moDCs with anti-human APC-MHC-II (HLA-DR) antibodies. Values represent the means of the MFI values from, at least, 6 independent assays.

In the previous section (section 4.1.1), we studied the sialyltransferases involved in human moDC sialylation and highlighted the significant contribution of ST3Gal-1 and ST6Gal-1 (Videira et al., 2008). Now, having observed that sialidase treatment induced DC maturation, **we wished to further inspect the type(s) of sialylation whose shortage was (or were) involved in this maturation process by focusing on ST3Gal-**

1-deficient and ST6Gal-1-deficient DCs. To address that question, we assayed *ex vivo* DCs from mice deficient in either these sialyltransferases for a more mature phenotype. For that, we quantified the expression of MHC II and CD86 in DCs present in blood, lymph nodes and spleens of ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ mice. The MHC II expression was higher in ST6Gal-1$^{-/-}$ DCs when compared with WT mice, but no apparent differences were observed regarding CD86 expression (Table 4.2). ST3Gal-1$^{-/-}$ mice presented insignificant variation of the MHC II and CD86 expression (Table 4.2).

To determine whether the increased expression of MHC II in ST6Gal-1$^{-/-}$ DCs could be linked to one particular DC subset, we focused our analysis on the lymphoid, plasmacytoid, and conventional DC subsets, based on the DC expression of CD8α, B220/CD45RA and CD11b markers, respectively (Table 4.2). Upon examination of each of these subsets for the expression of MHC II and CD86, we noted that the increased MHC II expression – detected in the total ST6Gal-1$^{-/-}$ DCs – was mainly present in the plasmacytoid (B220^{+}) DC subset (Table 4.2). Moreover, no specific sialylation absence showed an impact on the expression levels of the co-stimulatory marker CD86 in all the analyzed tissues' DCs. These results suggest that the more mature phenotype found in DCs from ST6Gal-1$^{-/-}$ mice, was mainly related – but not limited – to the plasmacytoid DC subset.

These last observations and the observations initially described on this section on sialidase-treated human moDCs after endocytosis, led us to clarify the contribution of ST3Gal-1- and ST6Gal-1-mediated sialylation on those previous observations. In practical terms, we wished to confirm the increased intensity of MHC II in bone marrow-derived DCs (BMDCs) from ST3Gal-1$^{-/-}$ or ST6Gal-1$^{-/-}$ mice before and after endocytosis. Bone marrow cells were used due to its higher generation yield, when compared to an *ex vivo* isolation from mice, as previously mentioned (subsection 1.1.1.2.1). Generally, as expected from the *ex vivo* DC analysis, ST6Gal-1$^{-/-}$ BMDCs showed a higher expression of MHC II than WT (Fig. 4.5A). However, contrasting with the analysis patent

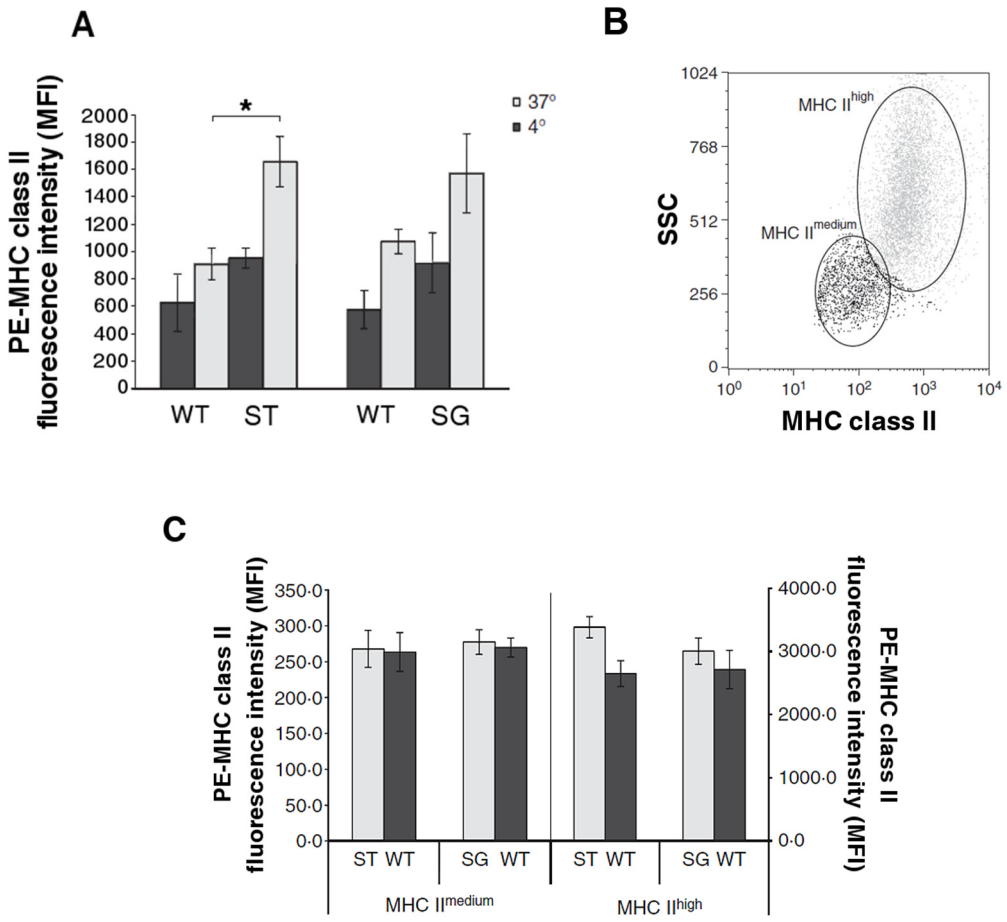
in table 4.2, ST3Gal-1$^{-/-}$ BMDCs showed a significant and even higher expression of MHC II (Fig. 4.5A).

Table 4.2 – Maturation analysis through expression of major histocompatibility complex class II (MHC II) and CD86 in ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ mouse dendritic cells, compared with wild-type control, in blood, spleen and lymph nodes. The values are expressed in mean ± SEM of mean fluorescent intensity (MFI) units, as defined by the flow cytometer. DCs were considered CD11c^{+} and MHC II^{+} cells. N indicates the number of mice used for each determination. [1]Values are related to total DCs. [2]Values related to plasmacytoid (B220+) DC subset.

		N	MHC II[1]	MHC II (B220^{+} DCs)[2]	CD86[1]
Blood	WT	8	157.0 ± 40.7	117.3 ± 20.6	22.78 ± 0.55
	ST3Gal-1$^{-/-}$	6	236.0 ± 59.5	166.0 ± 60.2	20.19 ± 1.79
	ST6Gal-1$^{-/-}$	10	280.4 ± 37.9 * p = 0.046	272.2 ± 26.9 *** p = 0.0005	26.55 ± 2.01
Spleen	WT	6	575.9 ± 61.4	784.8 ± 94.0	66.55 ± 5.37
	ST3Gal-1$^{-/-}$	7	554.0 ± 140.5	654.2 ± 92.5	65.65 ± 5.23
	ST6Gal-1$^{-/-}$	11	903.1 ± 91.0 ** p = 0.009	1047.0 ± 93.8	62.75 ± 7.28
Lymph nodes	WT	7	975.6 ± 105.8	864.3 ± 153.0	209.4 ± 47.60
	ST3Gal-1$^{-/-}$	6	1465.0 ± 366.8	1197.0 ± 171.0	198.0 ± 14.40
	ST6Gal-1$^{-/-}$	9	1498 ± 74.5 ** p = 0.007	1810.0 ± 177.0 ** p = 0.003	245.0 ± 44.50

To investigate whether the increased MHC II expression was exclusively derived from the endocytosis or if it was present, originally, in the ST6Gal-1$^{-/-}$ and ST3Gal-1$^{-/-}$ BMDCs, we have analyzed the MHC II expression in the control BMDCs which had not undergone endocytosis. The intensity of MHC II fluorescence also tended to be higher in both control ST6Gal-1$^{-/-}$ and ST3Gal-1$^{-/-}$ BMDCs than control WT BMDCs (Fig. 4.5A, 4°C condition). After endocytosis (Fig. 4.5A, 37°C condition), either WT, ST3Gal-1$^{-/-}$ or ST6Gal-1$^{-/-}$ BMDCs presented two distinguishable BMDC subpopulations based on the mean fluorescence intensity of MHC II (MHC II^{medium} and MHC II^{high}) (Fig. 4.5B). When gating both subpopulations and comparing the intensity of MHC II fluorescence, the MHC II^{medium} subpopulation was approximately identical, in the three analyzed BMDCs. **However, the**

MHC II^{high} population of both ST6Gal-1$^{-/-}$ and ST3Gal-1$^{-/-}$ BMDCs presented a slightly increased intensity of MHC II fluorescence (Fig. 4.5B and C). The percentage of MHC II^{high} subpopulation of BMDCs compared to the MHC II^{medium} subpopulation was about 10% and 5% higher in ST6Gal-1$^{-/-}$ and ST3Gal-1$^{-/-}$ BMDCs than in their WT homologues, respectively, but the differences were not statistically significant (Fig 4.5D and E)). Regarding the corresponding endocytosis values of these populations, the MFI of FITC-labelled ovalbumin of the MHC II^{medium} subpopulation was approximately identical, in the three analyzed BMDC types, whereas the one from the MHC II^{high} population of both ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ BMDCs presented a decreased intensity [49% (p = 0.0075) and 78% of fluorescence intensity relative to WT, respectively] (Fig 4.5F and G). Collectively, **these data indicate that BMDCs deficient for ST6Gal-1 or ST3Gal-1, upon endocytosis, show an augmented number of fully mature BMDCs subpopulation with decreased endocytosis capacity**.



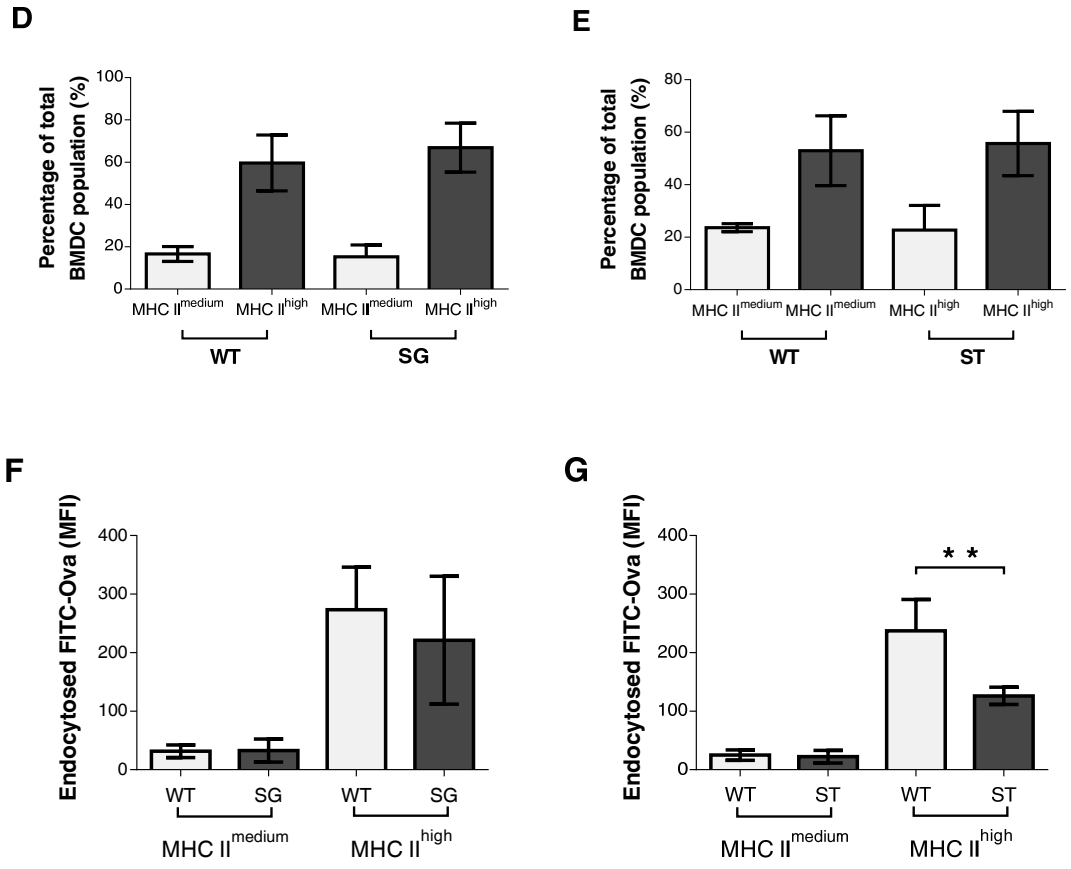


Fig. 4.5 - Major histocompatibility complex (MHC) expression is increased in ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ bone marrow-derived dendritic cells (BMDCs). The MHC II expression of ST3Gal-1$^{-/-}$ (ST) and ST6Gal-1$^{-/-}$ (SG) BMDCs was analyzed by staining with PE-anti I-A^{b}, after exposure to FITC-conjugated ovalbumin and incubation at 37°C or 4°C, as described in the Materials and methods. Control assays were performed in parallel with wild-type (WT) BMDCs. (A) The results are expressed as the mean fluorescence intensity (MFI) ± SEM of ST3Gal-1$^{-/-}$ BMDCs (six mice) and ST6Gal-1$^{-/-}$ BMDCs (seven mice). Significantly different values, related to WT BMDC, were observed at 37°C for the ST3Gal-1$^{-/-}$ BMDCs (* p<0.05). (B) Scatter plot showing that, after endocytosis (at 37°C), BMDCs express different levels of MHC II, defining a MHC II^{medium} and a MHC II^{high} subpopulation. The data shown correspond to analysis of one representative population of BMDCs (either from WT, ST3Gal-1$^{-/-}$ or ST6Gal-1$^{-/-}$ mice) acquired by flow cytometry. (C) MHC II^{medium} subpopulations of ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ BMDCs are no different from WT BMDCs in terms of MHC II expression, while MHC II^{high} subpopulations showed increased MHC II expression. The results are the means ± SEM of the MFI for MHC II for the MHC II^{medium} and MHC II^{high} subpopulations, gated as described in (B). The left axis corresponds to the MHC II^{medium} subpopulation and the right axis corresponds to the MHC II^{high} population. (D and E) After ovalbumin endocytosis, the MHC II^{high} BMDC subpopulation from ST6Gal-1$^{-/-}$ (ST) (D) mice or ST3Gal-1$^{-/-}$ (SG) (E) mice is larger than its WT counterpart. The results are expressed as the mean of the percentage of the different BMDCs subpopulations relatively to the total BMDCs ± SEM of ST3Gal-1$^{-/-}$ BMDCs (N≥5), ST6Gal-1$^{-/-}$ BMDCs (N≥5) and WT BMDCs (N≥5). (F and G) The amount of ovalbumin endocytosed by the MHC II^{high} subpopulations is also lower than the one from its WT homologues. Significantly different values, related to WT BMDC, were observed at 37°C for the ST3Gal-1$^{-/-}$ BMDCs (** p<0.01). The

results are expressed as the mean fluorescence intensity (MFI) ± SEM of ST3Gal-1$^{-/-}$ BMDCs (N≥5), ST6Gal-1$^{-/-}$ BMDCs (N≥5) and WT BMDCs (N≥5).

4.1.3.1 Discussion

With the recent progress of glycobiology it is becoming evident that sialic acid is relevant to the immune responses and the evaluation of sialylation-modification at the leucocyte surface contributes to a better understanding of the immune functions. We have previously demonstrated that the expression of surface sialylated structures was modulated during moDC differentiation and maturation, and we suggested that changes in sialylation are related to specific DC functions (Videira et al., 2008). Nevertheless, because of the characteristic complexity of DCs, the identification of the specific nature and role of these structures is still difficult to predict.

Here we show that the sialylation shortage, generated by sialidase treatment, induces an increased expression of MHC molecules and co-stimulatory molecules at the cell surface of human moDCs. To obtain a better insight, we studied DCs that were deficient for ST3Gal-1 and ST6Gal-1. As mentioned before, these two sialyltransferases are probably the most relevant for moDCs sialylation, they are significantly expressed by human moDCs and have a good correlation between their mRNA expression levels and the α2,3- and α2,6-sialylation profile during differentiation and maturation process of human DCs (Videira et al., 2008). In addition, **the data derived from these mouse models with ST6Gal-1 or ST3Gal-1 deficiencies suggest that the overall extent of cell surface sialylation, and not of specific sialylated glycoprotein entities, may be the important contributing factor, hinting at a possible synergy of several cell surface receptors**.

In this study, as part of this possible synergistic effect, specific differences in DC maturation and physiological distribution were noted between ST6Gal-1$^{-/-}$ compared to ST3Gal-1$^{-/-}$ and WT animals (see Table 4.2). These differences may indicate functional specializations for sialic acid linkages specified by the two sialyltransferases, which, in

their turn, may be dependent on the presence of several cell surface molecules as targets for sialylation or as recognizants of sialylated structures. Indeed, human moDCs express several receptors, such as Siglecs (see section 1.3.1), that recognize sialic-acid-containing glycans, and may have preferences for either the Sia(α2,3) linked to Galβ1,3GalNAc or the Siaα2,6 to Gal(β1,4)GlcNAc structures synthesized by ST3Gal-1 or ST6Gal-1, respectively (Varki and Angata, 2006;Varki and Gagneux, 2012). Siglecs have the potential to interact not only *in trans* with sialylated ligands found in other cells, but also *in cis* with ligands found at the same cell surface (Varki and Angata, 2006;Varki and Gagneux, 2012). The physiological functions of the majority of Siglecs are still unknown. However, because they contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), they are expected to have a major role in controlling immunity. Furthermore, additional glycan-recognizing receptors exist, and removal of the sialic acid residues, normally residing as the outermost residue of glycans, may uncover ligands for other immunologically relevant carbohydrate-binding molecules such as galectins. Some members of galectins, a family of lectins that recognize β-galactosyl-containing glycoconjugates, have been implicated in both innate and adaptive immune responses (Stowell et al., 2008). The biological functions of the galectins expressed by DCs are probably complex and are not well understood. Interestingly, it was found that galectins (namely galectin-1 and -9) could, indeed, induce DC to mature, with important adhesion properties modulated in the case of galectin-1 (Dai et al., 2005;Fulcher et al., 2006;Zhuo and Bellis, 2011). In this scenario, it is difficult to anticipate the extent of molecules and mechanisms that are engaged when DCs are treated with sialidase.

One point that should be addressed is the possibility that the observed DC maturation could be the result of the engagement of the innate receptors because the sialidase, used throughout this study, was of bacterial origin and could be contaminated with microbial products, particularly endotoxins. Nevertheless, to rule out that possibility, control assays were performed with a sialidase, whose activity was inactivated by heat, preserving eventual endotoxins present.

Considering the fact that differential sialylation can influence the accessibility of MHC epitopes to antibodies that recognize them (Liberti et al., 1979;Boog et al., 1989), it would be possible that the increased antibody binding to MHC molecules, observed in sialidase-treated DCs, was the result of an enhanced antibody affinity rather than of augmentation of the number of binding sites of such molecules. However, it should be noted that the increased immunogenicity of sialidase-treated DCs is confirmed not only by the increased binding of anti-MHC antibodies, as shown by flow cytometry, but also at functional level (transcription of specific cytokines and T cell priming proficiency) as it will be shown in the following section 4.4. In addition, qualitative differences in the extent of antibody binding to MHC II molecules were also observed among ST6Gal-1$^{-/-}$ and ST3Gal-1$^{-/-}$ DC subsets, corroborating the idea that clearance of cell surface sialic acids is indeed involved in an elevated expression of MHC II at the cell surface.

Our findings strongly suggest that **sialic acid linkages mediated by different sialyltransferases have distinct influence on diverse, fundamental DC functions, such as DC maturation. More specifically, sialic acid deficiency, either in ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ BMDCs or in human sialidase-treated moDCs, triggers DC maturation**.

4.1.4 Intracellular activation pathways of DCs and potentially involved sialylated receptors

Whether desialylation is naturally present (due to sialyltransferases deficiencies in gene expression, deletion or glycan moiety shedding) or artificially induced (by sialidase-mediated cleavage), we have previously observed (in subsection 4.1.3) that it induces mechanisms of maturation in DCs. We then asked **whether induced DC maturation**

was a consequence of the signaling transduction induced by cell membrane, sialylated receptors.

In mammalian species, the **M**itogen **A**ctivated **P**rotein **K**inases (**MAPKs**) signaling transduction pathway is involved in all aspects of the immune response, and cell death when immune function is complete (Dong et al., 2002;Nakahara et al., 2006). **The MAPK signaling pathway is a highly conserved pathway that is involved in diverse cellular functions, including cell proliferation, cell differentiation and apoptosis**. After recognition of PAMPS by PRRs, it transmits activating or inhibitory signals via phosphorylation from the cell surface down to their cytoplasmic substrates, being also translocated into the cell nucleus, where many MAPK targets, such as transcription factors, are found. This pathway comprises three major signaling pathways: the **E**xtracellular signal-**R**egulated **K**inases 1 and 2 (**ERK 1/2**) or p44 pathway, the **p38** pathway and the c-**J**u**n k**inase (**JNK**) pathway (Chang and Karin, 2001;Johnson and Lapadat, 2002), all of them known to be involved in the maturation and allostimulation mechanisms.

Particularly, **MAPKs have been shown to be involved in DC maturation** (Sato et al., 1999;Arrighi et al., 2001;Agrawal et al., 2003;Nakahara et al., 2004). Thus, given the importance of this signalling pathway, we used a two-step approach: first, we sought to clarify which of the intracellular signalling pathways was activated following sialic acid removal by performing western blots of the major MAPK; second, we determined the potentially involved cell-surface receptors using the data obtained in the previous step and crossing it with MALDI-TOF/TOF mass spectrometry of SNA lectin binding (α2,6-sialylated) receptors. This α2,6-sialylation linkage was chosen given the *Clostridium perfringens* sialidase (used throughout out studies) preference for the Siaα2,6Gal substrate (Bouwstra et al., 1987). Other sialidases with other substrate preferences (such as the Siaα2,3Gal linkage) could be used, such as sialidase from *Salmonella*

typhimurium. However, given our interest in the receptors affected by the *C. perfringens* sialidase action, we purified and analyzed the α2,6-sialylated ones.

We focused our study on the activation of ERK 1/2 signalling pathways. The MAPK respond to a wide range of stimuli (including regular cell handling), thus care was taken to reduce the activation level of these enzymes (as described in the *Materials and Methods* section of this dissertation). Western blotting techniques, using antibodies with specificities for phosphorylated (active form) and native ERK 1/2, were used to determine the amount of activated and total MAPKs. Using the soluble fraction of lysates of immature, sialidase-treated human moDCs, **we consistently observed a higher level of ERK 1/2 activation in sialidase treated moDCs, when compared with the positive and negative controls or the inactivated sialidase ('mock')-treated moDCs** (Fig. 4.6). Interestingly, even in the presence of the inactivated enzyme with no assessable sialylation reduction, the activation level was higher than the negative control (untreated cells) (Fig. 4.6B). These results confirm that **sialic acid absence induces MAPK signalling pathway, namely, the ERK pathway**, although the induction of other pathways should not be discarded.

After confirmation of the receptor-mediated induction of maturation, we sought to find the receptors potentially affected by sialidase treatment and subsequently involved in the maturation induction. For this purpose, we performed mass spectrometry analysis of α2,6-sialylated proteins isolated by SNA lectin affinity chromatography from immature moDCs lysates. After successful separation by SNA lectin affinity chromatography, the samples were analyzed by MALDI-TOF/TOF, with posterior data mining of the obtained results. The resulting list of α2,6-sialylated glycoproteins, presented in the Annex I (section 7), shows a variety of cell surface targets of sialidase action, both cytoplasmic and membranar, and it includes proteins involved in metabolism, gene expression, signalling, antigen presentation and actin organization. But, most interestingly, are the presence of sialylated proteins that are described as triggering intracellular signalling

mechanisms, such as α_M-integrin (CD11b) and β_2-integrin (CD18), commonly integrated in **mac**rophage**-1** antigen (**Mac-1**) (also known as **c**omplement **r**eceptor **3** (**CR3**)). The next step in the determination should be, hence, the isolation of these integrins, confirm its sialylation status and functional relation, in DCs, with the ERK signalling pathway or others.

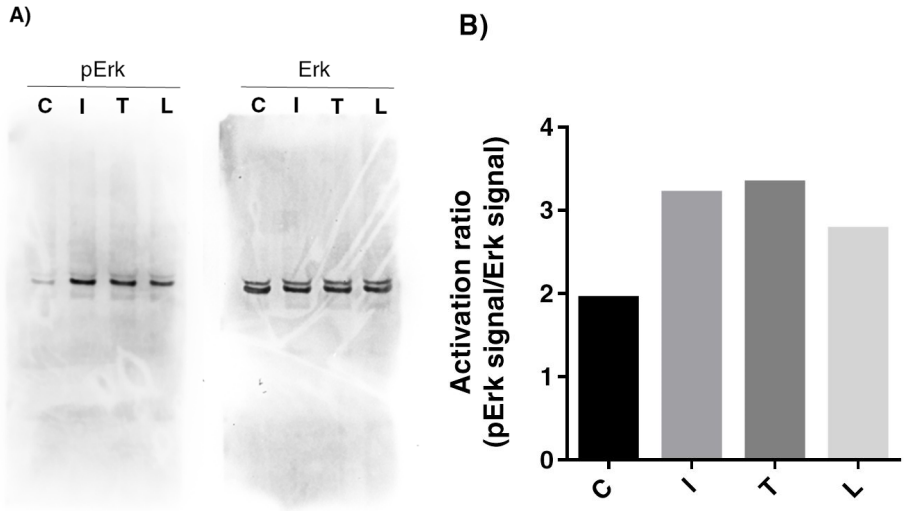


Fig. 4.6 – Expression of ERK 1/2 and phosphorylated-ERK 1/2 MAPK in moDCs. ***A)*** The ERK 1/2 activation level of moDCs was determined by Western blot analysis of soluble phase of lysates of moDCs of different conditions: C – control (untreated) (negative control); I – inactivated sialidase-treated ('mock'-treated); T – sialidase treated; L – LPS-stimulated moDCs (positive control). The left blot image is obtained by incubation of the membrane with the separated, transferred proteins with anti-phosphorylated-ERK 1/2 (pErk) antibody, whereas the right blot image is the same membrane, after pErk antibody removal, incubated with anti-total ERK 1/2 antibody, thus giving the total intracellularly available amount of ERK 1/2. This image is representative of, at least, four (4) assays. ***B)*** Using the image acquisition software, the blot density could be translated into a numeric value, which was used to calculate the presented activation ratio given by the reason between the 'pErk' and the corresponding 'Erk' blot density values, for each of the conditions tested.

4.1.4.1 Discussion

The maturation mechanisms are a key feature of the DC immunobiology. As previously mentioned, maturation marks a new phase in the DC lifecycle: from a "passive", information collecting phase characterized by high endocytic potential and constant environment screening, to an "active", stimulatory phase. This new phase is mainly characterized by upregulation of the antigen presenting and co-stimulatory molecules, inflammatory cytokines' secretion and homing to the lymph nodes.

Simultaneously, the obtained antigenic information is processed and the cytoplasm and cell membrane is rearranged (adopting its hallmark dendrites). Maturation is normally initiated after recognition of PAMPs or other “foreign”/“non-self” antigens by PRRs or other immune receptors such as Fc receptors or complement receptors. Recognition of antigens includes the intracellular translation of this recognition by a cascade of signals, such as the MAPK pathways. These pathways affect many processes in DC immunobiology: for instance, on mature DCs, p38 and JNK are involved in increased surface antigen expression, inflammatory cytokine production and allostimulatory capacity; the ERK signaling pathway positively regulates inflammatory cytokine production and shows a tendency to negatively regulate IL-12 production and the phenotypic maturation of moDCs. (Puig-Kroger et al., 2001;Nakahara et al., 2006).

Observations have previously been made regarding on the impact of desialylation in monocytes’ intracellular signaling. Desialylation of glycoconjugates on the surface of purified monocytes led to activation of ERK 1/2 (Stamatos et al., 2004a). The results here obtained, regarding MAPK activation on moDCs, follow the same trend and clearly point to the activation of signal translation after change of the glycosidic charge of DC surface proteins. After exogenous sialic acid removal by an α2,6-selective sialidase, the observed maturation is associated with a clear increase of the phosphorylated (activated) form of ERK 1/2. This increase may justify some of the changes induced by sialidase action given the functional impact ERK has in DC immunobiology, namely the increase in IL-6 and slight increase of TNF-α and IL-1β. In DCs, evidence has also been produced showing that the various signalling pathways are differently elicited in a stimuli-dependent way (Karakhanova et al., 2010) inducing different effects. This also raises the hypothesis that sialidase-treatment may specifically induce maturation mechanisms via other MAPK signalling pathways other than ERK 1/2. In order to confirm these hypothesis, studies of sialidase treatment-induced maturation in the remainder MAPK pathways (namely, p38 and JNK) in the presence of selective MAPK inhibitors should be performed. A hypothetical p38 and JNK activation may account for the remainder

maturation features, namely, for the marked mature phenotype changes observed. Considering that p38 has a higher impact in DC immune functions (Nakahara et al., 2006), one might speculate that even a small variation in p38 activation could be nonetheless sufficient to override the slightly negative influence caused by ERK activation, or even being synergistically needed. Such cases have been observed in other contexts: given two apparently opposed signals transmitted via ERK 1/2 and p38, the latter only prevailed in the presence of the apparently antagonistic ERK signal (Karakhanova et al., 2010).

The most amazing feature of the MAPK signalling pathways is the fact that, knowing the vast variety of receptors that potentially induce the signalling pathways, all the signals elegantly converge to one MAPK, and from that point onwards, are divergently translated in a large variety of translocated nuclear transcription factors (Fig. 4.7). Still, knowing the type of induced signalling pathway may valuably hint at the number and type of receptors involved in the studied phenomenon.

Using an overlapping/complementary reasoning, we decided to determine the potential targets of sialidase by mass spectrometry, using state-of-the-art MALDI-TOF/TOF after lectin affinity separation. Crossing the results represented in Annex I (section 6) with the receptor families described as inducing ERK and p38 signalling pathways lead us to focus our attention on the α_M- (CD11b) and β_2- (CD18) integrins. It is well established that integrins depend on a conformational change in order to change to their active form, leading, among other effects, to intracellular signalling cascades. CD11b and CD18 integrins dimerize to form the complement receptor CR3, known to be expressed on DCs (Reis et al., 2007;Li et al., 2011), being the dominant phagocytotic complement receptor (Sandor et al., 2013). $\alpha_M\beta_2$ integrins bind an array of ligands that do not share canonical binding sequences and are expressed exclusively in leukocytes with functions in adhesion, especially during the formation of the immunological synapse, transendothelial migration of immune cells and interaction with the extracellular matrix (Humphries et al., 2006;Kinashi, 2007;Smith, 2008).

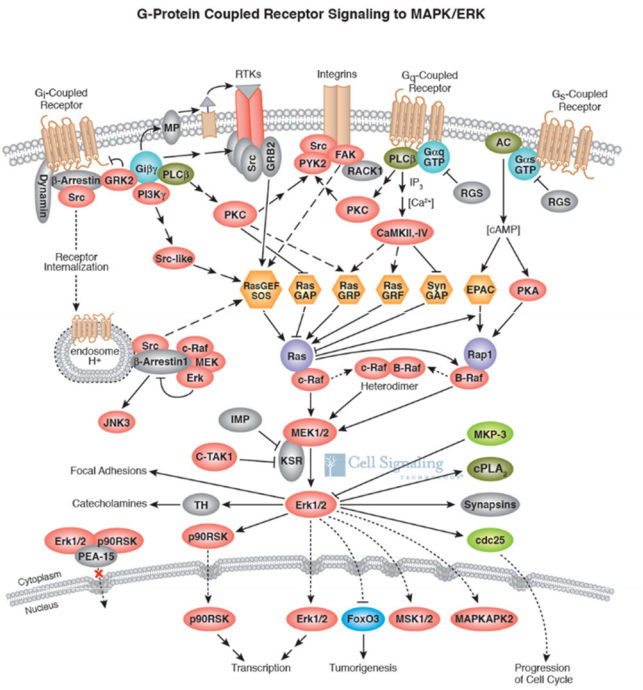


Fig. 4.7 – ERK 1/2 MAPK intracellular signalling pathway, from a G-Protein Coupled Receptor starting point. The figure depicts the major signalling cascades featuring direct/indirect (full line/dotted line, respectively) stimulation or inhibition (full-line, arrow point or blunt point, respectively). *In Cell Signalling Technology, Inc. website (*http://www.cellsignal.com/common/content/content.jsp?id=pathways-mapk-g*).*

Integrins need to be activated by intracellular signals derived from the activation of other ITAM-bearing cell surface receptors or chemokine receptors, in what is known by "inside-out" mode, thus acquiring the correct conformation for functional binding (Kellermann et al., 2002;Takagi and Springer, 2002). However, in a not totally clear mechanism, known to require the ***Syk*** tyrosine kinase (Abram and Lowell, 2009), **integrin binding to ligand also induces an "outside-in" activation, triggering intracellular signalling cascades leading to ERK activation** (Whitlock et al., 2000;Streuli, 2009). Hence, it is reasonable to suppose that **CD11b and CD18 integrins can be likely candidates through which sialic acid absence/removal may have a crucial impact in DC immune functions**. Nevertheless, future studies should be carried out to confirm this hypothesis, and consolidate sialic acid's position as an important immune modulator.

4.2 Sialylation influence in DC endocytosis and phagocytosis

4.2.1 Endocytosis

DCs use several pathways to capture antigens including macropinocytosis, receptor mediated endocytosis and phagocytosis. The former is described as being exclusive of DCs and activated macrophages but is not present in monocytes (Sallusto et al., 1995). The latter two require cell-surface receptors such as C-type lectins, mannose receptors, Fc receptors or integrins, with phagocytosis also uptaking large molecules or cells (Banchereau et al., 2000;Norbury, 2006). In order to verify whether the presence of sialic acid was critical for the endocytosis performed by monocytes and moDCs, we have tested the uptake capacity for three endocytic agents after treating both types of cells with sialidase. Ovalbumin, Lucifer Yellow and dextran are all described, in DCs, as being uptaken by macropinocytosis, although a number of receptors have also been identified as involved in the internalization of ovalbumin (Kikuchi et al., 2005), whereas dextran is uptaken mainly through mannose receptor (Sallusto et al., 1995). In this work, we observed that **monocytes treated with sialidase present an increased capacity to uptake ovalbumin and dextran, when compared with non-treated monocytes**, as determined by a higher mean fluorescence intensity (MFI), (1.3- and 2-fold, respectively) when analyzed by flow cytometry (Fig. 4.8 and Table 4.4). The uptake of Lucifer Yellow by monocytes was almost negligible and unaffected after sialidase treatment. By contrast, when compared with the controls, sialidase-treated moDCs showed a decrease in the capacity to uptake all the endocytic tracers tested (Fig. 4.8). According to the MFI analysis, after sialidase treatment, the uptake of ovalbumin, Lucifer Yellow and dextran is significantly decreased by 0.76, 0.75 and 0.85-fold, respectively (Table 4.4). We have also determined the cell percentage which underwent endocytosis in the assays performed with the different endocytic tracers and found no significant differences after treating the cells with sialidase (Table 4.4).

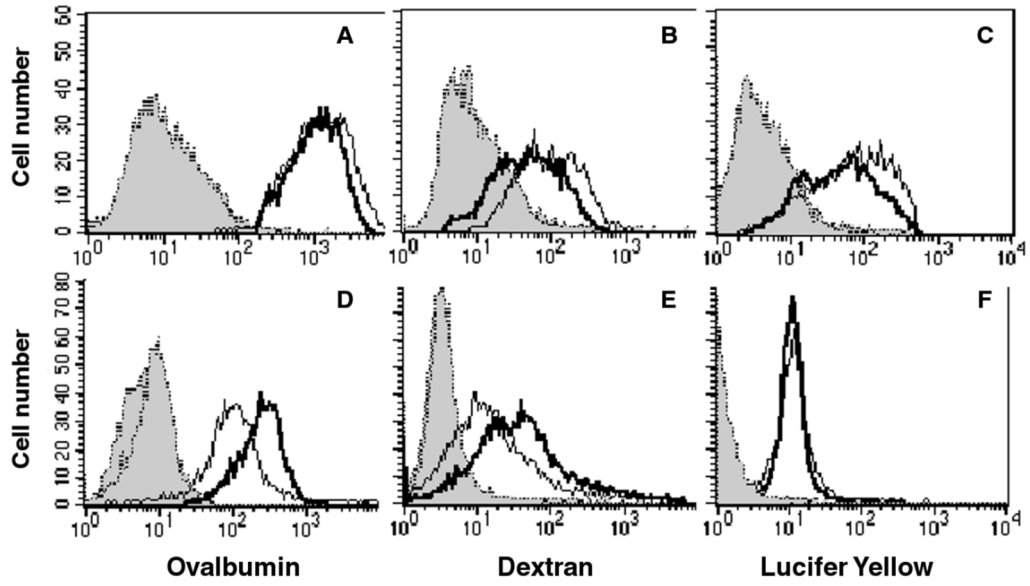


Fig. 4.8 - Effect of sialidase treatment on the uptake capacity of immature moDC (upper panels) and monocytes (lower panels). Cells were incubated for 1 h with ovalbumin (a, d), dextran (b, e) and Lucifer Yellow (d, f), as described in Materials and methods and analyzed by flow cytometry. Bold and solid lines represent, respectively, sialidase treated and non-treated cells incubated at 37°C. Grey histograms represent control cells incubated at 0°C. Data shown are a representative of one of different experiments, whose statistical analysis is represented in Table 4.4.

Table 4.4. Statistical analysis of the effect of sialidase treatment on the endocytosis capacity of moDC and monocytes. Values are the mean fluorescence intensity (MFI) and percentage (%) of fluorescent cells and represent the mean ± SD of at least 4 independent assays. The values from the corresponding control cells incubated at 0°C were subtracted.

		Sialidase-treated		**Non treated**		**Ratio§**	
		MFI	**%**	**MFI**	**%**	**MFI**	**%**
MoDC	*Ovalbumin*	1239.3 ± 150.4	68.1 ± 8.4	1638.0 ± 231.1	70.1 ± 5.2	0.76**	0,98
	Lucifer Yellow	203.3 ± 34.0	32.4 ± 7.1	285.9 ± 45.2	37.6 ± 7.6	0.75*	0,86
	Dextran	319.7 ± 45.2	35.4 ± 3.8	372.1 ± 46.2	35.6 ± 5.9	0.85***	0,99
Monocyte	*Ovalbumin*	134.3 ± 20.5	79.0 ± 1.7	98.9 ± 18.5	80.0 ± 2.7	1.35*	0,98
	Lucifer Yellow	11.0 ± 1.9	87.0 ± 2.1	10.1 ± 1.8	90.0 ± 1.2	1.10	0,96
	Dextran	94.7 ± 1.9	80.0 ± 6.2	46.5 ± 1.6	86.5 ± 3.3	2.00***	0,92

§The ratio corresponds to the values from sialidase treated cells divided by the values of non-treated cells. *p< 0.05; **p< 0,01;***p< 0,005.

A possible explanation for this observation is that, as we've previously seen, sialidase treatment triggered DC maturation, a process known to be accompanied by diminished antigen uptake. The results supporting this hypothesis were previously mentioned in section 4.1.3, where we explored the relation between sialidase treatment and maturation.

Since we demonstrated that, at least, *ex vivo* ST6Gal-1$^{-/-}$ DCs presented increased expression of MHC II (as patent in section 4.1.3), we then asked whether ST6Gal-1$^{-/-}$ or ST3Gal-1$^{-/-}$ DCs also had impaired endocytic capacity. To answer this, similar to the described in subsection 4.1.3, we used DCs differentiated *in vitro* from bone marrow collected from mice deficient for these STs.

We performed FITC-conjugated ovalbumin endocytosis assays with ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ BMDCs. As a control condition, similar assays with WT BMDCs were always run in parallel. The percentage of BMDCs that endocytosed ovalbumin was roughly identical to that of WT BMDCs (± 62%). However, and most notably, the fluorescence intensity of FITC-labelled ovalbumin was reduced in BMDCs from ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ mice [61% (P = 0.0078) and 76% of fluorescence intensity relative to WT, respectively] (Fig. 4.9). These observations demonstrate that ST3Gal-1$^{-/-}$ and, to a lesser extent, ST6Gal-1$^{-/-}$ BMDCs endocytose less ovalbumin than WT BMDCs.

The observations here presented confirm the previously obtained data using sialidase-treated moDCs. However, we did not check the endocytosis performance of ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ monocytes. The data would have allowed us to give a deeper insight on the specific sialylation influence on the different endocytic processes on monocytes.

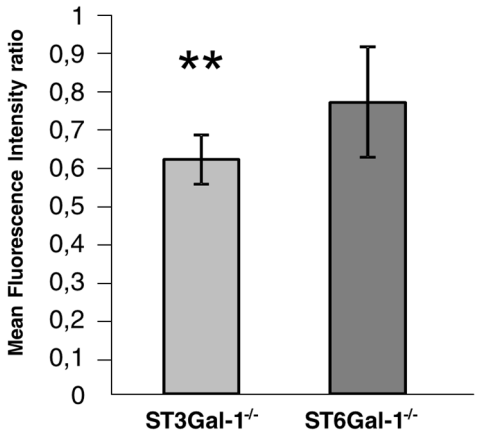


Fig. 4.9 - Endocytic ability of ST3Gal-1$^{-/-}$ and ST6Gal-1$^{-/-}$ bone marrow-derived dendritic cells (BMDCs). The ST3Gal-1$^{-/-}$ or ST6Gal-1$^{-/-}$ BMDCs were incubated, for 30 min, with 0.05 mg/ml FITC-conjugated ovalbumin, as described in the Materials and methods. Control assays were performed in parallel with wild-type (WT) BMDCs. The mean fluorescence intensity (MFI) values obtained at 4°C were subtracted from the 37°C values. The results are expressed as the ratio of the MFI ± SEM of ST3Gal-1$^{-/-}$ BMDCs (N = 6) or ST6Gal-1$^{-/-}$ BMDCs (N = 7) related to WT BMDC. Significantly different values were observed for the endocytic capacity of ST3Gal-1-/- BMDC (**P < 0.01)

4.2.2 Phagocytosis

4.2.2.1 Desialylated moDCs and mature moDCs (m-moDCs) exhibit an improved phagocytic capacity for E. coli

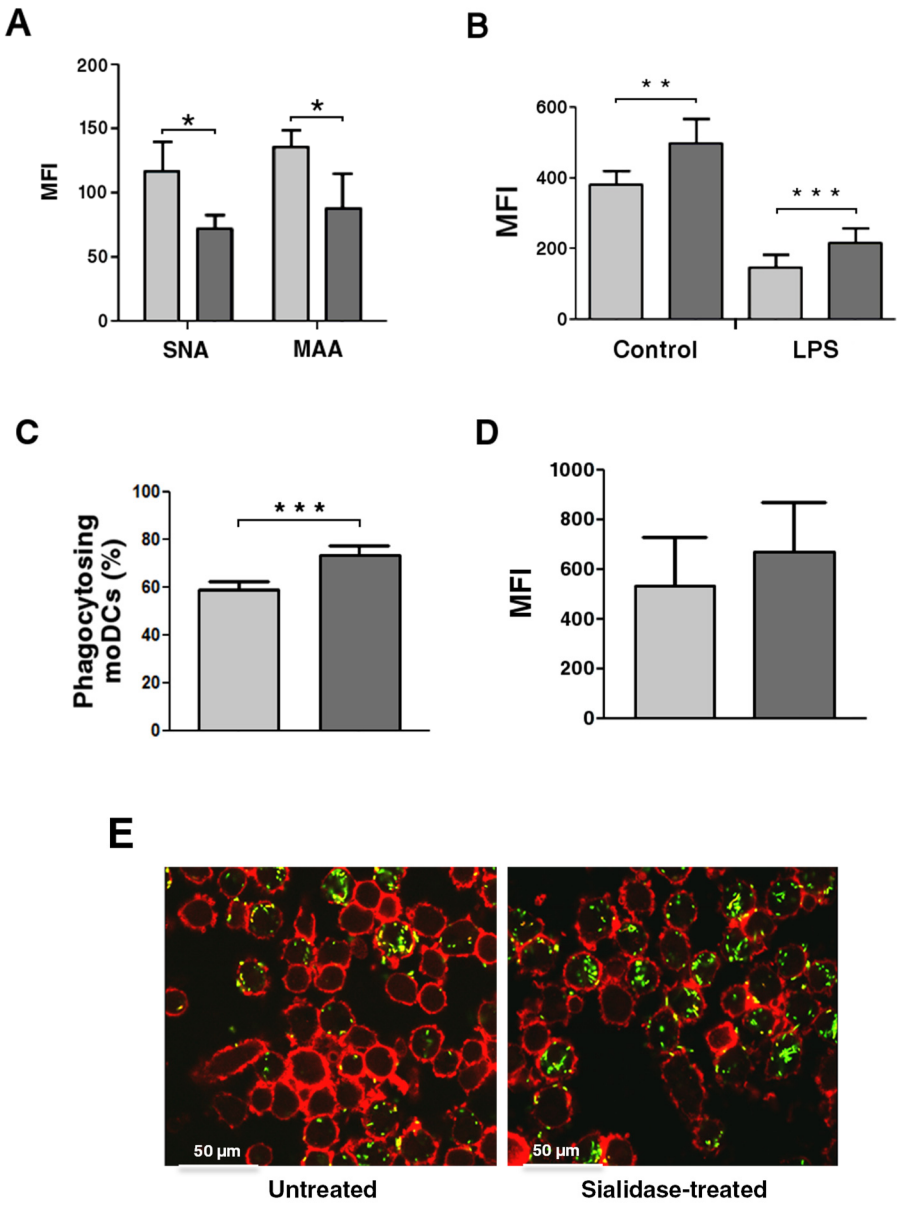
Phagocytosis is a major form of endocytosis, dedicated to the elimination of whole bacteria and/or viruses and, thus, with greater visibility and impact upon the clearance of pathogens. Although DCs are not professional phagocytic cells, like macrophages, they are still able to phagocytose, with this endocytosis type being a major antigen source to process and present them to T cells. As such, to complete the study of the aforementioned endocytic processes, **we sought to study the implications of sialic acid mediating phagocytosis**.

As previously performed with endocytosis, in order to better investigate whether removal of cell surface sialic acids affected phagocytosis, we analyzed the capacity of sialidase-treated moDCs to internalize a K12-derived strain of *E. coli*. After confirmation of successful α2,6- and α2,3-desialylation of moDCs (by SNA and MAA staining, respectively) (fig. 4.10C), flow cytometry analysis revealed that **desialylated moDCs phagocytosed significantly more *E. coli*** (MFI = 499.90 ± 66.92) **than fully sialylated moDCs** (MFI = 382.80 ± 38.94) (Fig. 4.10A) – **a fact contrasting with the previously found reduction in DC endocytic levels**. **Sialidase intervention also resulted in an increase in the number of moDCs that internalized bacteria** (73-74% ± 3.9 as compared with 58-92% ± 3.8 in the control assays) (Fig. 4.10D).

An established hallmark of DC maturation is the loss of internalization capacity (Sallusto et al., 1995;Garrett et al., 2000), so, as expected, pre-stimulation of moDCs with LPS decreased phagocytosis in ~60% (Fig. 4.10A). To assess the effect of sialidase treatment in already mature moDCs (m-moDCs), we desialylated LPS-stimulated m-moDCs. However, **sialidase treatment significantly lowered the typical decrease in phagocytic capacity of LPS- or TNF-α-matured moDCs** (Fig. 4.10A and 4.10E). The

observed differences in phagocytosis were exclusively the result of antigen internalization and could not be attributed to differences in adhesion of the antigen to the cell surface, based on quenching experiments with trypan blue and negative control assays performed at 4°C. Analysis of phagocytosis by confocal microscopy further confirmed the intracellular localization of the fluorescent bacteria (Fig. 4.10B and 4.10F).

Taken together, these observations, therefore, suggest that **phagocytosis can be altered by the removal of sialic acids regardless of the maturation state of the DCs**.



Green - *E. coli*; Red - actin filaments

F

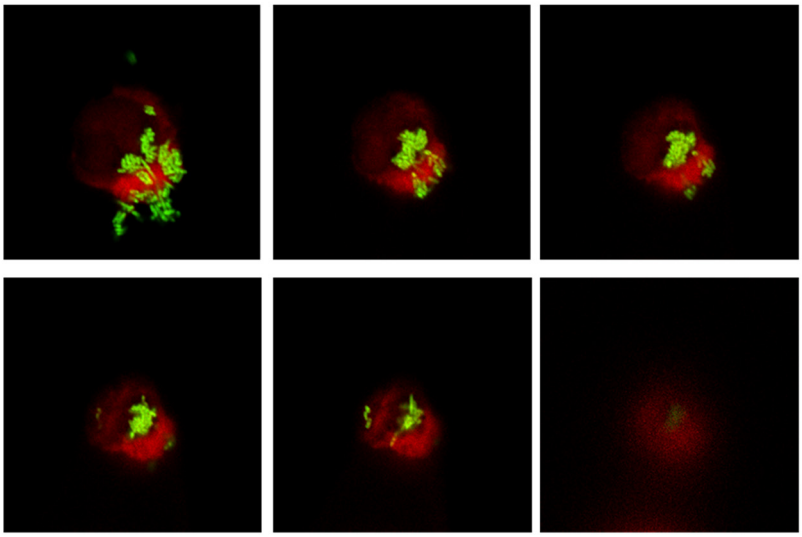
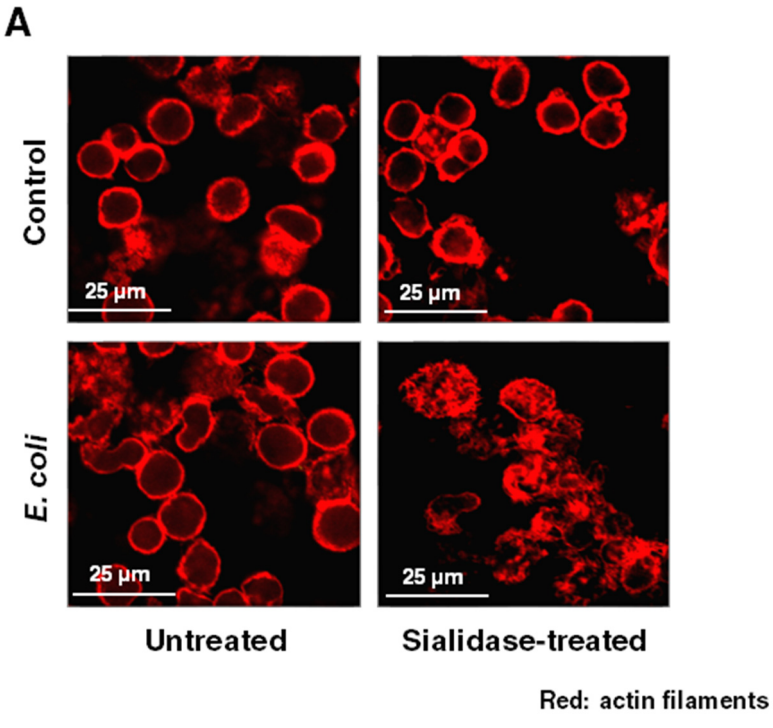


Fig. 4.10. Sialidase treatment improves phagocytosis by human monocyte-derived dendritic cells (moDCs) and mature moDCs (m-moDCs). Immature moDCs and lipopolysaccharide-matured (LPS) m-moDCs were treated with sialidase (dark gray bars on A, B, C and D) or left untreated (light gray bars on A, B, C and D), following incubation with fluorescent *Escherichia coli*, for 1h at 4°C or 37°C. (A) moDCs were stained with *Sambucus nigra* lectin (SNA; recognizing α2,6-sialic acids) and *Maackia amurensis* lectin (MAA; recognizing α2,3-sialic acids) lectins following sialidase treatment and analyzed by flow cytometry. Values represent the means of the MFI of at least three independent assays. Statistical significance (*$p < 0.05$) refers to the difference between untreated and sialidase-treated moDCs. (B) The phagocytic capacity by moDCs and m-moDCs was evaluated by flow cytometry as the mean fluorescence intensity (MFI) and values obtained at 4°C were subtracted. Values represent the means of at least 20 independent assays. Statistical significance (**$p < 0.001$ or ***$p < 0.0001$) refers to the difference between untreated and sialidase-treated moDCs or m-moDCs (C) Sialidase treatment improves the % of human moDCs that phagocytosed *E. coli*. moDCs were treated with sialidase or left untreated, following incubation with fluorescent *E. coli*, for 1 h at 37ºC. The phagocytic capacity by moDCs was evaluated by flow cytometry as the % of DCs that phagocytosed *E. coli*. Values represent the means of at least 10 independent assays. Statistical significance (***P<0.001) refers to the difference between untreated and sialidase treated moDCs. (D) Sialidase treatment improves phagocytosis by human TNF-α-mature moDCs. TNF-α-matured m-moDCs were treated with sialidase or left untreated, following incubation with fluorescent *E. coli*, for 1h at 37°C. The phagocytic capacity by moDCs was evaluated by flow cytometry as the fluorescence (MFI). Values represent the means of at least 4 independent assays. (E) Representative confocal microscopy images showing moDCs with internalized *E. coli* (green). Actin filaments of the moDCs' cytoskeleton were stained with Phalloidin Alexa Fluor 633 (red). (F) Representative confocal microscopy images of detailed Z-staking series of a moDC with their cytoskeleton stained with Phalloidin Alexa Fluor 633 (red) showing internalized *E. coli* (green). moDCs were previously incubated with fluorescent *E. coli*, for 1 h at 37ºC.

4.2.2.2 *Desialylation, moDC cytoskeleton organization and the activation of Rho GTPases upon E. coli stimulation*

Depending on the receptors involved, phagocytosis often requires significant remodeling of the actin cytoskeleton and the activation of small GTPases of the Rho family, namely Cdc42 and Rac1 (Garrett et al., 2000;Shurin et al., 2005). After observing that desialylation improves phagocytosis, we asked **whether desialylation could affect the cytoskeleton organization or increase the level of Cdc42 and Rac1 GTPases activation**. As shown in Fig. 4.11A, desialylation did not affect the cytoskeleton organization of moDCs. However, in the presence of *E. coli*, these visibly desialylated moDCs showed a disorganization of actin filaments with a tendency to aggregate and form clusters (Fig. 4.11A). Sialidase treatment did not affect the basal level of active Cdc42 and Rac1 GTPases in moDCs (Fig. 4.11B). However, no increase in the activation of Cdc42 and Rac1 upon *E. coli* phagocytosis was observed when the moDCs were sialidase-treated (Fig. 4.11B). Together these data indicate that **increased phagocytosis upon removal of cell surface sialic acids by sialidase was not the result of improved moDC cytoskeleton dynamics**.



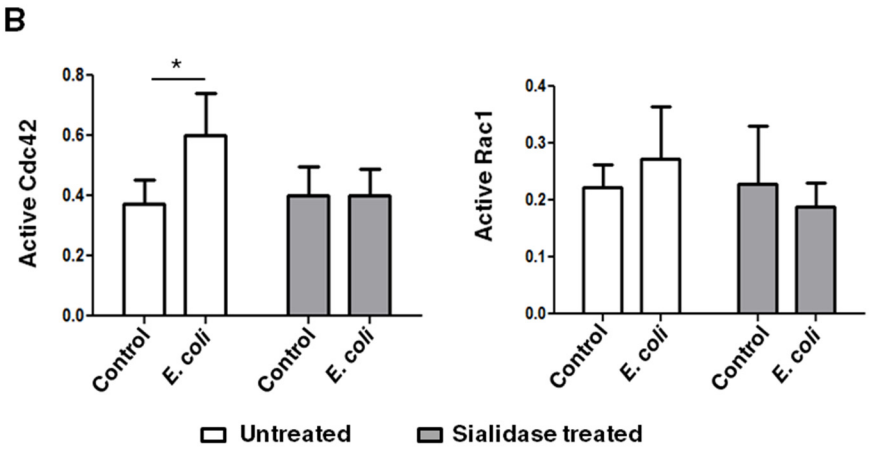


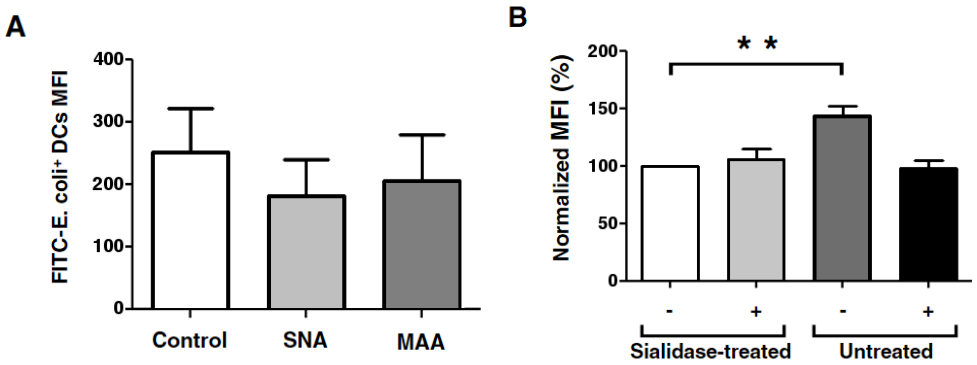
Fig. 4.11. Sialidase affects the cytoskeleton organization and the activation of Rho GTPases. Monocyte-derived dendritic cells (moDCs) were treated with sialidase or left untreated, and then incubated or not (control) with *Escherichia coli* for 15 min at 37°. (A) The moDC cytoskeleton was stained with Phalloidin Alexa Fluor 633 (red) and analyzed by confocal microscopy. (B) The Rho GTPase activation level was measured in moDC lysates, as described in the *Materials and methods* section. The level of active Rac1 or Cdc42 (in arbitrary units) is based in optical density values obtained at 490 nm after subtracting negative control values. Values represent the means of at least four independent assays. Statistical significance (*P < 0.05) refers to the difference between control moDCs and moDCs incubated with *E. coli*.

4.2.2.3 Enhanced phagocytosis mediation by sialic acid moieties

To further understand **how moDCs cell surface sialic acids influence phagocytic activity**, we started by examining the capacity of desialylated moDCs to internalize *E. coli* in the presence of structural blockers. After hindering α2,6-linked and α2,3-linked sialic acids with SNA and MAA lectins, respectively (as an alternative to cleavage of sialic acids moieties by sialidase), the *E. coli* phagocytosis showed a slight tendency towards inhibition (Fig. 4.12B). These results suggest that **the improved phagocytosis requires the removal of sialic acid rather than the simple masking of cell surface sialic acid by lectins**. As such, if covalently linked cell surface sialic acids are, indeed, involved in modulation of phagocytic activity, we predicted that restoration of sialic acids should undermine the sialidase activation of phagocytosis. For that matter, we studied how moDCs phagocytosed *E. coli* in the presence of free sialic acid. Interestingly, **the enhanced phagocytosis observed in sialidase-treated moDCs was completely abolished in the presence of the unbound sialic acid** (Fig. 4.12A). This result was

largely reminiscent of previous observations reported by our group regarding the presence of a moDC cell surface sialyltransferase activity (Cabral et al., 2010). The presence of such a re-sialylation mechanism could, thus, mediate the reconstruction of cell surface sialic acid linkages in the presence of the donor substrate cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-5-NeuAc). Accordingly, we tested if, in the presence of CMP-5-NeuAc, the phagocytosis enhancement in desialylated moDCs would revert to physiological levels. As expected, **the presence of CMP-5-NeuAc abolished the improvement of *E. coli* phagocytosis by desialylated moDCs** (Fig. 12C) through the observed restoration of moDC surface sialylation, thus substantiating the idea that **sialic acids act as phagocytic modulators**.

A sialic acid-dependent mechanism for the binding of sialylated bacteria to desialylated immune cells has been reported (Avril et al., 2006;Carlin et al., 2009;Khatua et al., 2010). As the K12-derived *E. coli* strain used in the present work contains sialic acids, we then wished to determine if improved phagocytosis was dependent on the *E. coli* surface sialic acids. As shown in Fig. 4.12D, **desialylation of *E. coli* re-established the phagocytosis to levels similar to the control ones suggesting a mechanism of phagocytosis requiring the presence of sialic acids in both moDC and *E. coli***.



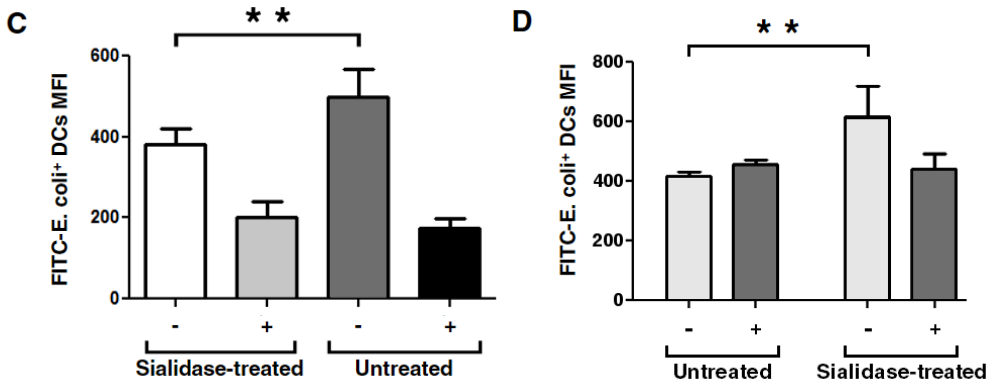


Fig. 4.12 - *Escherichia coli* phagocytosis is influenced by sialic acid moieties. Monocyte-derived dendritic cells (moDCs) were treated with sialidase or left untreated, and then incubated or not (control) with E. coli for 1 h at 4°C or 37°C. (A) The effect of hiding surface a2,6-linked and a2,3-linked sialic acids in phagocytosis was determined by incubating untreated moDCs in the absence (control) or in the presence of *Sambucus nigra* lectin (SNA) or *Maackia amurensis* lectin (MAA) blocking lectins. Values represent the means of MFI of at least three independent assays. (B) The influence of free sialic acid on phagocytosis was determined by evaluating the phagocytic capacity of moDCs in the presence (+) or absence (-) of this sugar. Values, representing phagocytosis, calculated as the percentage (%) of the mean fluorescence intensity (MFI; obtained by flow cytometry) normalized with respect to untreated moDCs incubated without free sialic acid. Values represent the means of at least five independent assays. Statistical significance (**P < 0.001) refers to the difference between sialidase-treated and untreated moDCs. (C) The influence of ectosialyltransferase activity in phagocytosis was assessed by conducting the assay in the presence (+) or absence (-) of the sialyltransferase substrate, cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-5-NeuAc). Values represent the means of MFI of at least five independent assays. Statistical significance (**P < 0.001) refers to the difference between sialidase-treated and untreated moDCs. (D) The participation of sialic acids from *E. coli* surface on phagocytosis was determined by comparing the capacity of moDCs to internalize *E. coli* when sialidase-treated (+) or left untreated (-). Values represent the means of MFI of, at least, three independent assays. Statistical significance (**P < 0.001) refers to the difference between sialidase-treated and untreated moDCs.

4.2.2.4 Phagocytosis of pathogenic E. coli isolates

We then asked **whether moDC desialylation could improve the internalization of pathogenic strains of *E. coli***. We used different pathogenic *E. coli* isolates (designated here as I, II, III, IV) obtained from urine cultures and hemocultures of patients either with a urinary infection or septicemia. As with their non-pathogenic homologues, **after treating moDC with sialidase, the phagocytosis of all four pathogenic isolates was enhanced**. In fact, as in the case of the K12-derived strain, an improvement of 29%,

29%, 17% and 30% in the phagocytosis of pathogenic isolates I, II, III and IV, respectively, was observed when moDCs were treated with sialidase (Fig. 4.13). **These results suggest not only a role for sialic acid as a modulator of phagocytosis by moDCs, but also a potential therapeutic utility against pathogenic *E. coli* infections**.

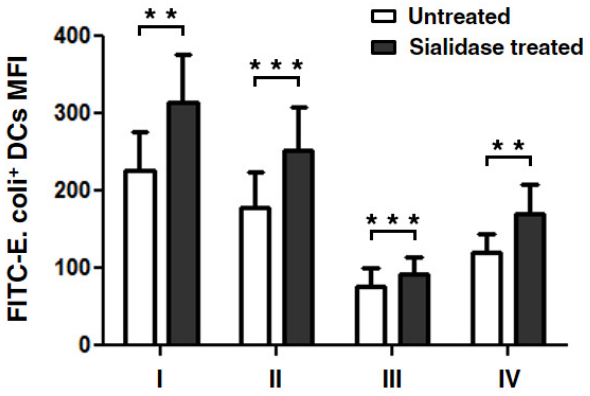


Fig. 4.13 – Sialidase treatment improves the capacity of monocyte derived dendritic cells (moDCs) to phagocytose pathogenic *Escherichia coli* isolates. moDCs were sialidase-treated or left untreated and incubated for 1h, at 4°C or 37°C, with pathogenic *E. coli* isolates (I, II, III, IV). The phagocytic capacity was evaluated by flow cytometry as the MFI and values obtained at 4° were subtracted. Values represent the means of, at least, 10 independent assays. Statistical significance (**$p < 0.001$ or ***$p < 0.0001$) refers to the difference between sialidase-treated and untreated moDCs.

4.2.2.5 Improved phagocytic capacity for E. coli of ST6Gal-1$^{-/-}$-mouse BMDCs

To further document the correlation between cell surface sialic acid and the phagocytic capacity of DCs without using sialidases, we used BMDCs harvested from sialyltransferase ST6Gal-1$^{-/-}$ mice. As shown in Fig. 4.14, ST6Gal-1$^{-/-}$ BMDCs showed an approximately twofold improvement in their capacity to phagocytose *E. coli* versus BMDCs from the WT mice (Fig. 4.14A). An increase of almost twofold on the percentage of DCs was also observed (Fig. 4.14B). These results corroborate the data from human desialylated moDCs, linking phagocytic capacity with cell surface sialylation status. Furthermore, the data suggest **a specific contribution of α2,6-sialic acids to this process**.

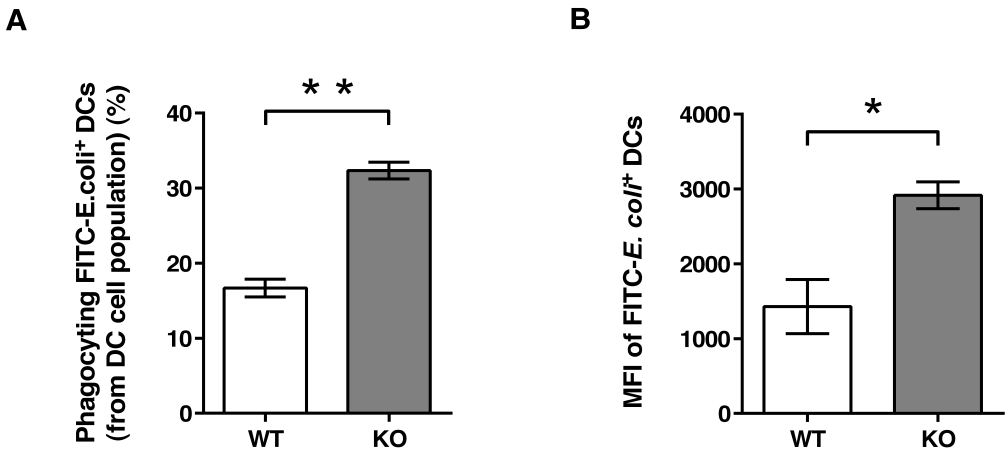


Fig. 4.14 – BMDC phagocytosis of *E. coli*. After incubation of BMDCs with FITC-labelled *E. coli*, as described in Material and Methods, the phagocytosis performance of BMDCs from ST6Gal-1$^{-/-}$ (KO) and wild-type (WT) mice was evaluated by flow cytometry and compared. Two parameters were quantified and are above represented: (**A**) the percentage of the BMDC population (FITC^{+} CD11b^{+} CD11c^{+} I-A^{b+} cells) that effectively phagocytosed *E. coli*; and (**B**) the efficiency of DCs in *E. coli* phagocytosis, given by the MFI of the FITC^{+} DC population. Values represent the means of four (4) experiments. Significantly different values were observed regarding the phagocytic capacity of KO BMDC (* $p < 0.05$) and the fraction of phagocytosing KO BMDCs (** $p < 0.01$).

4.2.3 Discussion

In our experiments, the endocytic capacity of both monocytes and moDCs was affected when the surface sialic acid was removed by sialidase treatment. In the case of monocytes, the treatment gives rise to an increased fluorescence intensity of the cells, suggesting an increased amount of endocytosed particles by cell; contrarily to the moDCs, whose treatment decreases slightly the amount of endocytosed particles. Either monocytes or moDCs are able to endocytose dextran and ovalbumin through several mechanisms, which include phagocytosis and endocytosis via different groups of receptor families, such as Fc receptors, Toll-like receptors and C-type lectin receptors (Geijtenbeek et al., 2004). Nevertheless, both cells present distinct distribution of endocytic receptors, with DCs presenting a higher content and also specific receptors

such as DC-SIGN and DEC-205 (Jiang et al., 1995;Bleijs et al., 2001). In DCs, there is also the additional contribution of macropinocytosis, the actin-dependent formation of large vesicles for fluid-phase solutes uptake. Macropinocytosis is absent in monocytes, which is in accordance with the negligible Lucifer Yellow endocytosis, observed in the monocyte endocytosis assays (Fig. 4.8). Since the sialic acid removal affects the uptake capacity in an opposite manner in monocytes and moDCs, **it could be suggested that the reduction in sialylation affects negatively an uptake mechanism exclusive of moDC, such as the macropinocytosis, but on the other hand, it promotes a further uptake mechanism probably shared by monocytes and moDCs**. Considering three facts, namely: 1) the results obtained using sialidase-treated human moDCs and the ones obtained using ST-deficient mice; 2) that during the maturation of DCs its endocytic capacity decreases; and 3) that ST6Gal-1 and ST3Gal-4 enzymes are significantly downregulated during maturation (see section 4.1.1.2, Fig 4.3C and E), suggests **these enzymes may be involved in the DC endocytosis**. The downregulation of these STs could be associated with the decreased expression of sialylated molecules involved in the endocytosis, or, in case of equivalent expression, to the decreased sialylation of such molecules leading to its lower activity and, ultimately, to poor DC endocytosis. Nevertheless, the information regarding the glycosylation of molecules involved in the antigen uptake is still scarce and further studies remain to be performed to better understand the role of sialic acid in signaling a specific endocytosis mechanism.

Antibiotic resistance among Gram-negative pathogens has been on the rise and the current gold-standard for the treatment of bacterial infections – the use of antibiotics – is under serious threat (Slama, 2008). Pathogenic variants of *E. coli* are known to easily acquire resistance, especially for antimicrobial agents that have long been in use in humans. Hence, while the search for new antibiotics continues, there is an urgent need for effective combat strategies against risk-associated Gram-negative bacteria.

Reinforcement of the immune system through a synergy of cellular stimulation, anti-microbial action, and long-lasting antibacterial protection is considered an interesting choice. Through their potent immunoregulatory capacities, **DCs are promising therapeutic targets to boost sufficiently robust and long-lasting immunity against pathogenic bacteria.** Therefore, understanding the mechanisms that modulate the pivotal DC–pathogens interaction is, nowadays, a fundamental goal.

Our results, patent on this thesis, and other recent reports show that human moDCs are highly sialylated (Julien et al., 2006;Bax et al., 2007;Trottein et al., 2009), and a growing body of evidence has implicated a role for their sialylated structures in the modulation of moDC functions (Silva et al., 2012), including endocytosis mechanisms. Here, **we addressed the question of whether moDC sialic acids are particularly relevant for phagocytosis**. Physiologically, it is already accepted that cell surface sialic acid content can be modulated by endogenous sialidases such as Neu1 (Amith et al., 2010) and by sialidases from exogenous sources released by pathogenic bacteria or viruses during the course of an infection. Regarding phagocytosis, it was reported that mouse cell surface Neu1 activates phagocytosis by macrophages and DCs through desialylation of cell surface receptors (Seyrantepe et al., 2010). In addition, surface desialylation by influenza virus sialidase stimulates the internalization of target virus infected cells by mouse macrophages (Watanabe et al., 2004).

In this section, we reported for the first time that **the phagocytosis by human moDCs could also be modulated by cell surface sialic acids**. We demonstrated that **sialidase treatment significantly improved *E. coli* phagocytosis regardless of the state of DC maturation**. As sialidase treatment led to actin cytoskeleton disorganization and did not lead to increased activation of the Rho family of GTPases, we inferred that **desialylation influenced phagocytosis through an actin-independent mechanism**. However, further investigations are necessary to fully understand the supportive mechanisms.

We showed that **the enhanced phagocytosis induced by desialylation was inhibited by free sialic acid and was dependent on *E. coli* sialic acids**. Moreover, this phenomenon was not observed by simply hiding cell surface sialic acid by the use of lectins, hinting that actual removal of cell surface sialic acid residues was needed for phagocytosis improvement. Concordantly, **phagocytosis improvement could be reversed by regeneration of cell surface sialic acid linkages by means of an ectosialyltransferase activity present on DC surfaces**. Of note, we also found that **desialylation also improved the capacity of moDCs to internalize pathogenic *E. coli* isolates**, suggesting the sialidase mechanism to be ubiquitous regarding other *E. coli*. According to the data shown above, **this mechanism should be restricted, at least, to bacteria that express cell surface sialylated structures**. However, further studies are necessary to elucidate if this mechanism is *E. coli* restricted or could be extended to other sialylated Gram-negative bacteria.

One possible hypothesis to explain the sialidase phenomenon **is the engagement of receptors that become more accessible after sialidase treatment**. This mechanism hypothesis is supported by previous suggestions that sialylation content, modulated by Neu1 sialidase, is a new important parameter controlling interactions between macrophage receptors, their ligands and signaling proteins (Seyrantepe et al., 2010).

Bacterial pathogen-associated molecular patterns (PAMPs) are recognized by a variety of pathogen-recognition receptors (PRRs), which include several sialylated receptors and receptors that recognize sialylated structures. Toll-like receptors (TLRs) recognize several microbial ligands, leading to the activation of intracellular signaling cascades. Interestingly, endogenous or exogenous sialidases have an essential role in LPS-induced TLR4 activation by cleaving specific sialic acid residues thus making PAMPs recognizable (Stamatos et al., 2004b;Amith et al., 2010;Stamatos et al., 2010). Sialic acid binding immunoglobulin-like lectins (Siglecs) are receptors that specifically

recognize sialic acids and are involved both in endocytosis and cellular signaling functions (Lock et al., 2004). Siglecs are usually masked by *cis* interactions with sialic acids expressed on the same cell membrane, which can be unmasked following exposure to sialidase or, in some cases, by cellular activation (Crocker et al., 2007). Most Siglecs possess cytoplasmic tails harboring immunoreceptor tyrosine-based inhibitory motifs (ITIMs) with an important immunoregulatory role. Indeed, some Siglecs from the CD33-related family can suppress the TLR dependent production of TNF-α and IL-6 pro-inflammatory cytokines and enhance the production of the anti-inflammatory cytokine IL-10 (Ando et al., 2008). On a related note, many pathogens are known to possess, or acquire, sialic acid to interact with Siglec-expressing leucocytes thus advantageously modulating the immune response (Avril et al., 2006;Carlin et al., 2009;Khatua et al., 2010).

Taking these characteristics into account, and considering that *E. coli* exhibit some ligands for some TLRs and/or Siglecs receptors, **these are candidate receptors involved in the observed improvement of *E. coli* phagocytosis by desialylated moDCs**. Nevertheless, it is probable that a complex combination of receptors is simultaneously engaged following moDC desialylation resulting in the observed up-regulation of phagocytosis. Connecting with previous observations of this work, it has been reported that integrin $\alpha_M\beta_2$-mediated phagocytosis is Cdc42- and Rac-dependent (Caron and Hall, 1998). Considering this and also the fact that sialidase treatment of DCs involves the activation of Cdc 42 and Rac, which correlate with increased *E. coli* phagocytosis, the results described in section 4.1.4 (activation of moDCs' ERK 1/2 MAPK following sialidase treatment of these cells) lead us to add to the Siglec hypothesis, a functional model that includes sialic acid as a novel modulator for $\alpha_M\beta_2$ integrins function. On this model**, sialic acid removal would influence these integrins conformation/stability to a more avid conformation, leading to activation of Rac and Cdc42 and enhanced phagocytosis, and, downstream the signalling pathway,**

activation of ERK 1/2 accounting for the previously mentioned effects mediated by this MAPK.

Our studies with BMDCs deficient for ST6Gal-1, which mediates the synthesis of the α2,6-sialyl linkages, strongly implicates the influence of specifically α2,6-sialic acids in this process and further suggests a role for the expression of the ST6Gal-1 enzyme in regulating DC phagocytic activity. ST6Gal-1 is one of the most highly expressed sialyltransferases in human moDCs (see section 4.1.1.1 of this thesis). One of the best known products of ST6Gal-1 is the counter-receptor of CD22 Siglec on B cells (Ghosh et al., 2006). Besides the humoral responses, the maintenance of myeloid homeostatic balance (Nasirikenari et al., 2006;Jones et al., 2010b;Nasirikenari et al., 2010), T cell functionality (Amano et al., 2003), and integrin signaling (Woodard-Grice et al., 2008) are just a few of the physiological processes that also implicate ST6Gal-1 participation. The complete set of specific ST6Gal-1 acceptor substrates is still not identified in DCs but it is most likely to involve key α2,6-sialylated receptors or α2,6-sialylated ligands engaged in *cis* interactions with endogenous receptors.

There is a possibility of *E. coli* receptors or components actively participating and interacting with desialylated moDCs. This possibility is irrelevant because, in this work, we adopted an experimental design that involved exclusively heat-killed rather than live bacteria.

Summarizing, in contrast to classical maturation stimuli such as LPS, TNF-α or IL-1β (Sallusto et al., 1995), desialylation-triggered maturation does not dramatically abrogate endocytosis mechanisms such as macropinocytosis or receptor-mediated endocytosis: **human or mouse DCs with reduced sialic acid content retain around 70% of their endocytic capacity**. In terms of phagocytosis, the data from the present study indicates that **desialylation, and in particular depletion of α2,6-sialic acids, improves the capacity of human moDCs to internalize *E. coli*, independently of their maturation**

stage. Further studies are still required to illuminate the details of the involved mechanisms, but the present work lays the foundation insight for the importance of cell surface sialic acids of DC at the critical interface between bacterial challenges and the mobilization of host adaptive immune responses.

We believe that these findings are relevant in adding to the knowledge required for the establishment of efficient antibiotic substitutes and immunotherapies, which are particularly important in fighting antibiotic-resistant Gram-negative pathogens.

4.3 α2,6-sialylation is involved in DC homing to lymph nodes

DC homing could be described as a multi-step process, including (1) mobilization, (2) detachment, (3) interstitial migration, (4) entry into the afferent lymphatics, and (5) transit via lymph (Alvarez et al., 2008) (figure 4.15). Most of these steps have continuously being found to be strongly influenced by leukocyte glycosylation and the tissue glycocalyx. Glycan-mediated processes in leukocyte migration are well described in literature and found to be essential in this process. In the case of DCs, this aspect has recently started to be studied, with many glycan-mediated interactions being described, such as: the Lewis Y antigen-mediated interaction between DC-SIGN and ICAM-2 on endothelial walls (Garcia-Vallejo et al., 2008); the binding of the macrophage Mannose Receptor to CD44 via chondroitin sulfate side chains in this antigen or to sulfated oligosaccharides of blood group Lewis a and Lewis x and sulfated N-glycans of lutropin (Leteux et al., 2000;Salmi et al., 2013); and, most notably and common to most innate immunity leukocytes, the sialyl Lewis X (sLe^x) mediated interaction between PSGL-1 and P-/E-selectins (Robert et al., 1999a;Bonasio et al., 2006;Silva et al., 2011), a paramount interaction in the rolling and tethering stages of the initial homing/migration process in high endothelial venules in several tissues.

However, **the role of α2,6-sialylated N-glycans in the migration and/or homing of DCs is still unclear**. In order to clarify this, end-point DC homing *in vivo* assays using the murine model were performed. In the first group of assays we generally assessed DC homing to regional draining lymph nodes, after inflammatory stimulus. Secondly, we performed a more detailed study using adoptively transferred BMDCs.

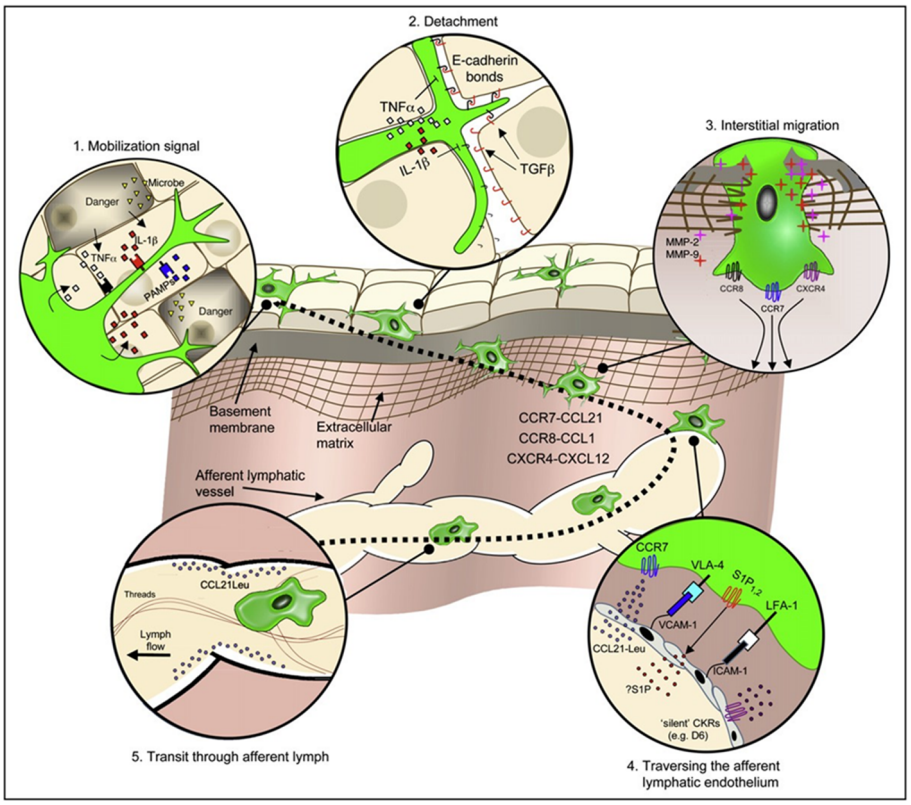


Fig. 4.15 – DC Trafficking in Peripheral Tissues. This schematic illustrates a proposed model for the interstitial migration for skin DCs from the cutaneous microenvironment to the afferent lymphatics *en route* to the LN. The migratory cascade is divided into five discrete steps (clockwise from top left), starting with recognition of a mobilizing signal (inset 1), detachment from structural tissue elements (inset 2), trafficking through interstitial space (inset 3), transit through the afferent lymphatic endothelium (inset 4), and transit through the afferent lymph vessels (inset 5). Major chemokine-chemokine receptor (CKRs) pathways and other trafficking molecules controlling DC migration are highlighted. *In Alvarez D. et al*., Mechanisms and Consequences of Dendritic Cell Migration, *Immunity, 29, 2008*.

In order to evaluate the α2,6-sialylation of N-glycans influence in DC migration on an inflammatory context, an Alexa 625-Ovalbumin + alum solution was intracutaneously injected in both WT and ST6Gal-1$^{-/-}$ mice scruff region in order to promote inflammation. Given that ovalbumin is essentially uptaken by DCs via endocytosis, the migrating (mature) DCs present in the draining lymph nodes will be, by this reason, fluorescently distinct. After 48h, the regional draining lymph nodes were collected and their resident cell population was analyzed by flow cytometry. The results, patent in figure 4.16A, clearly show **a reduced number of inflammatory DCs present in the lymph nodes of**

ST6Gal-1$^{-/-}$ mice when compared with their WT counterparts and hinted to a relevant role of α2,6-sialylation in DC homing.

Next, we wanted to evaluate if this functionality is dependent on the N-glycan's α2,6-sialylation of DCs or if it is a result of the presence of this type of glycosylation in adhesion molecules in the surrounding tissues to which DCs may bind. Our hypothesis was that **BMDCs derived from ST6Gal-1$^{-/-}$ mice would show impaired homing towards the lymph nodes**. To test this, LPS-stimulated and fluorescently-labelled BMDCs from WT and ST6Gal-1$^{-/-}$ mice were adoptively transferred to other mice, according to the conditions described in figure 4.16B.

The percentage of migrating DCs when there is a sialylation deficiency (ST6Gal-1 -/- deficient mice as donor, k/w and k/k conditions) is **significantly lower** (**approximately 50%**) than the percentage of WT BMDC (Fig. 4.16C). But, surprisingly, **the same significant reduction was also observed when there was α2,6-linked sialic acid absence *in the recipient mice***, being even more statistically significant ($p = 0.0049$ (w/k condition) and $p = 0.0051$ (k/k condition), Fig. 4.16C). A synergistic effect on the sialylation absence in both donor cells and recipient was not observed. As a control, labelled, immature BMDCs were also adoptively transferred, in parallel. Comparing the ratio of LPS-stimulated migrating BMDCs and unstimulated ones (Fig. 4.16D), (maturation control), the total number of DCs present in the regional draining lymph nodes in the former condition is higher.

Together, **these results point to a clear role of DCs' α2,6-sialylation of N-glycans in DC homing but it also suggests an equally importance of this sialylation type in adhesion ligands exogenous to DCs for functionally effective DC homing**.

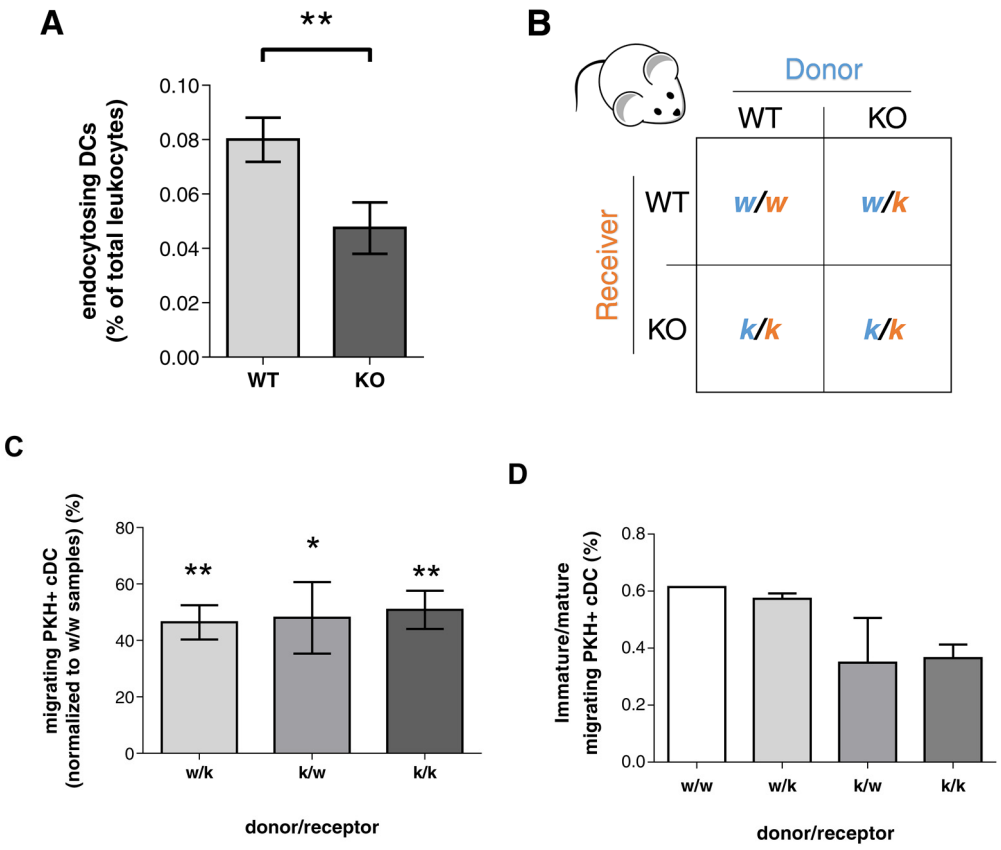


Fig. 4.16 – Migration assays of BMDCs. (A) *In situ* stimulation of inflammation-driven DC migration. After administration of an AlexaFluor 625-Ovalbumin + Alum suspension, the draining lymph nodes of the neck and thorax region were removed and the contained leukocytes collected, as described in Materials and Methods. The collected cells were then sorted by flow cytometry in order to evaluate the fraction of endocytosing DCs. The results are depicted as the wild-type (WT) or ST6Gal-1$^{-/-}$ (KO) DC percentage of total leukocytes collected and analyzed. (B) BMDC homing assay. Adoptive transfer of exogenously cultivated, stimulated and fluorescently labelled (PKH 67^{+}) BMDCs was performed as described in Material and Methods. represents the ratio of migrating BMDCs to total cDCs (CD11c^{+} CD11b^{+} I-A^{b+}), based on the data represented in B), present in the collected regional draining lymph nodes of the tested subjects, normalized to the same ratio in the control conditions. These conditions are given as the phenotype of the adoptively transferred BMDCs (donor) and the phenotype of the subject receiving the exogenously differentiated BMDCs (receptor): w/w (control condition, not represented, equal to 100%) – WT BMDCs transferred to WT receptor; w/k – WT BMDCs transferred to KO receptor; k/w – KO BMDCs transferred to WT receptor; k/k – KO BMDCs transferred to KO receptor. C) Immature to mature ratio of migrating BMDCs. The ratios were calculated between homologue conditions, viz. immature w/w to mature w/w, immature w/k to mature w/k, immature k/w to mature k/w and immature k/k to mature k/k.

Figures represent mean ± SEM of values corresponding to, at least, 5 independent experiments, except in C), which correspond to a minimum of 3 individual experiments. Significantly different values were observed in A) the migrated WT to KO BMDC (* $p < 0.05$), and B) the migrating BMDCs in the different adoptive transfer conditions (* $p < 0.05$, ** $p < 0.01$).

4.3.1 Discussion

While glycans have been implied in different aspects of DC migration, the relevance of α2,6-sialylated N-glycans in DC homing is still poorly understood. Here, we present evidence regarding the importance of this specific type of glycosylation in the DC adhesion and migration processes. **By showing a clear impairment of DC migration wherever an α2,6-sialylation defect is present, either on DCs or in the surrounding tissues, our results clearly show that α2,6-sialylation is relevant for DC homing to draining lymph nodes**. Thus, we suggest that **N-glycan α2,6-sialylation is a requirement for DC homing during inflammation**.

The natural question at this point is, hence, what are the N-glycosylated, α2,6-sialylated adhesion molecules and ligands involved in this mechanism, accounting for the observed approximate 50% reduction in DC homing.

Some of the previously mentioned α2,6-sialylated moDC receptors, as determined by mass spectrometry (see subsection 4.1.4 and section 6 for results) are also known to be involved in the ligation to extracellular matrix proteins or adhesion molecules, such as the $\alpha_M\beta_2$ integrin or the CD44 glycoprotein, and are known to be relevant players in DC homing mechanisms (Diacovo et al., 2005). Therefore, sialylation of these receptors may also be mediating DC adhesion to endothelium and homing.

In the terminal phase of myeloid cell differentiation, an increase of cell surface α2,6-sialylation that correlated with decreased adhesion to the bone marrow stroma was reported, in a mechanism thought to be mediated via $\alpha_M\beta_2$ integrin (Le Marer and Skacel, 1999). This principle of "**high sialylation, high mobility; low sialylation, high adhesiveness**" is also seen in other blood cell types and involving other receptors. For instance, removal of senescent erythrocytes seems to be dependent of a reduced sialic acid content of membranar proteins, namely CD44, leading to enhanced hyaluronan binding on the endothelium of tissues presenting high content of this glycan, namely liver

or spleen (Kerfoot et al., 2008). Lymphocytes, erythrocytes and platelets are also captured by endothelial galectins recognizing low sialic acid content in liver and spleen tissues (Schauer, 2009). As previously seen, α2,6-sialylated N-glycan content increases during monocyte to moDC differentiation conferring immature moDCs a high sialylation content. Once the maturation process begins, we observed a downregulation of ST6Gal-1 expression and activity.

It is known that sialidase-treated monocytes present enhanced adhesion to hyaluronic acid (Katoh et al., 1995;Katoh et al., 1999) and that sialylation loss, mediated by sialidase action or glycosylation shedding, is a requirement for integrin or CD44 mediated-leukocyte homing (Katoh et al., 1995;Katoh et al., 1999;Weber et al., 2004). Successful homing also depends on the firm arrest of leukocytes prior to transmigration via binding of adhesion molecules, such as β_2 integrins (LFA-1, CD11a,c,d/CD18) to ICAM-1, -2 or VCAM-1 (Butcher et al., 1999;Smith, 2008) (Fig. 4.15). Regarding these integrins, it is interesting to find that $CD18^{-/-}$ mice present a degree of DC migration impairment towards the lung tissue (approximately, 68%) (Schneeberger et al., 2000) **similar to the one here presented by us**.

A high sialic acid content in adhesion molecules may prevent DCs transmigration through lymphatic endothelium, with compromised homing towards the lymph nodes. Oppositely, **excessive low sialylation levels (such as in the case of ST6Gal-$1^{-/-}$ BMDCs) could lead to excessive firm adhesion to extracellular matrix, reducing migration towards the lymph nodes**. The sialic acid removal would lead to increased integrin avidity, significantly slowing down posterior cell detachment after transmigration, thus disturbing the normal transmigration under physiological flow conditions. This hypothesis needs to be tested by other techniques, such as *in vivo* microscopy, in order to observe what happens to the BMDCs that present little to none α2,6-sialylated N-glycans.

The reduced WT BMDC migration observed when these BMDCs were transferred into ST6Gal-1$^{-/-}$ KO host may be due to the asialylated nature of the ECM. In addition, the endothelial glycocalyx, has been described as being tendentiously anti-adherent mainly due to the presence of terminal, negative-charged sialic acid residues in the glycan structure (Simionescu and Simionescu, 1986;Silvestro et al., 1994;Constantinescu et al., 2003). Activation of endothelia was proven to induce a glycocalyx modulation in order to become more adherent (Sabri et al., 2000). The leukocyte-endothelium firm arrest, mediated by integrins and their ligands seem also to be influenced by sialylation in some of the adhesion molecules. For instance, Van Kooyk and collaborators have shown that a sialylation increase of ICAM-2 was translated in poor adhesion to all its DC counter-receptors, namely, LFA-1 and DC-SIGN (Weber et al., 2004). VCAM-1, another relevant integrin ligand expressed in endothelial wall, indirectly depends on the presence of lysossomal Neu1 for its expression (Yogalingam et al., 2008). It has also been described that endothelial cells up-regulate ST6Gal-1 expression and N-glycan α2,6-sialylation of the expressed ICAM-1 and VCAM-1 upon inflammatory stimulation, suggesting a structural preparation of these adhesion molecules for firm arrest (Hanasaki et al., 1994). The effect of both these observations are somewhat equivalent: **a fine balance of sialidases and sialyltransferases are, therefore, paramount for proper function of these adhesion molecules, with the removal of any of these enzymes seeming to disturb normal leukocyte homing, either by lack of adhesion or by excessive one**.

Another hypothesis may be the functional impact of sialylation removal on chemokine receptor expression, namely on DC homing-associated chemokine receptors such as CCR7, CCR5, CXCR4 and CCR8 (Diacovo et al., 2005). Of these, CCR7 is of paramount importance for successful DC homing. Two of its corresponding ligands, CCL21 and CCL19, are expressed by the lymphatic endothelium, forming the main chemotactical gradient responsible for homing DCs towards the lymph nodes (Fig. 4.15). The sialylation of CCL21 has a known impact in moDC homing: ST8Sia-4 polisialylation of neuropilin-2

was shown to promote chemotactic migration of human moDCs toward the chemokine CCL21, but not to CCL19 (Bax et al., 2009;Rey-Gallardo et al., 2010;Rey-Gallardo et al., 2011;Rollenhagen et al., 2013). Although, this is not a case of N-glycosylation (Rollenhagen et al., 2013), little has been explored regarding the glycosylation (and sialylation) of chemotactical mediators, leaving an open door for a possible role of ST6Gal-1 in lymph node chemotaxis.

The knockout of ST6Gal-1 stops the glycosidic chain extension at a terminal Galβ1,6GlcNAc or N-acetyl-lactosamine (LacNAc) glycan on an N-glycan chain, the preferential acceptor substrate for this enzyme. **The exposed LacNAc residues thus become potential ligand for lectins specifically recognizing this carbohydrate, such as galectins**. Their recognition and binding are inhibited by α2,6-sialylation of recognizable LacNAc residues of N-glycans (Amano et al., 2003;Cummings and Liu, 2009;Zhuo and Bellis, 2011). It is described that galectins-1, -3, -8 and -9 show selectivity for cell surface ligands as well as extracellular matrix (ECM) protein ones (Ozeki et al., 1995;Levy et al., 2001;Nishi et al., 2003;Fulcher et al., 2006;Cummings and Liu, 2009;Fulcher et al., 2009). Galectin-1 is mainly present in the ECM and lymphoid endothelium (Ahmed et al., 1996;He and Baum, 2004), two crucial substrates in the migration process. Triggering DC maturation via inflammatory cytokine stimulus leads to an upregulation and enhanced expression of galectin-1 by endothelial cells (Baum et al., 1995;Dietz et al., 2000;Pereira et al., 2005). Thus, our previously observed enhanced maturation of sialic acid-deficient DCs could plausibly stimulate the local production of galectin-1 by DCs themselves and by surrounding tissues, with concomitant autocrine and paracrine action. In addition, given the highly availability of LacNAc ligands for these lectins in ST6Gal-1$^{-/-}$ BMDCs or recipients, **it is logical to assume that galectin-1 may bind DCs in excess numbers or avidity, sterically hindering integrins or other adhesion molecules thus possibly impairing DC homing thus delaying their**

homing to the lymph nodes, similar to the proposed by Avni and collaborators (Avni et al., 1998). In fact, data regarding *in vitro* migration of monocytes in the presence of galectin-1, -2, -3 and -4 has shown that these galectins are, in fact, inhibitors of migration (Paclik et al., 2011), with galectin-1 presenting clear anti-adherent properties on already galectin-adhered cells (Cooper et al., 1991;Elola et al., 2005). The known anti-inflammatory effect of galectin-1 (La et al., 2003;He and Baum, 2006;Norling et al., 2008;Ilarregui et al., 2009) clearly contributes to these anti-adhesion mechanisms by, among other effects, downregulating endothelial adhesion molecules. Additional assays regarding the galectin involvement in this process are, naturally, needed to enlighten this question.

ST6Gal-1 can also be expressed by the liver as a soluble form with catalytic properties as part of the acute phase response in an inflammation context (Lance et al., 1989;Appenheimer et al., 2003), and, given the presence of glycoforms that could act as targets for the action of this enzyme, it would be expected a possible sialylation of these N-glycans on sialic acid deficient BMDCs by soluble ST6Gal-1 from the WT hosts (the K/W assay condition on fig. 4.16) thus reverting to the control conditions, translatable in a normalization of the migrating DCs percentage. Nevertheless, if the sialylation occurred, it didn't translate in the restoration of a normal migration patterns. Given that a high systemic level of pro-inflammatory cytokines for a sustained period of time is needed in order to initiate soluble ST6Gal-1 secretion by the liver, it is likely that threshold level wasn't reached causing the external sialylation to not occur. As such, the administration of DCs in the presence of an inflammatory stimulus could be a condition that may be explored in future developments in order to clarify this issue.

Altogether, it is clear that these results provide new insight on the homing processes, especially concerning N-glycans' α2,6-sialylation. As standing on the cell interface with

the exterior, it doesn't come as a surprise the multiplicity of players mediating the interaction of this type of sialylation with adhesion molecules, endothelial tissue and ECM. In fact, illustrating (and adding) to this problem is the fact that different DC subtypes adhere with different affinities to the different ECM components (Kohl et al., 2007). The facts here presented raise several questions that will complete the general model (viz. adhesion molecules modulation by sialylation, galectin and Siglec involvement) and contributing to advances in the use of DCs as immunotherapy. Furthermore, the data here presented further stresses that prior to a practical, clinical use of moDCs in DC immunotherapy, migration/homing assays should be performed in order for optimization of function and ultimate therapy success in what it is, of late, the Achilles' heel of this form of therapy.

4.4 Sialylation role in triggering the adaptive response

4.4.1 Altered expression of cytokines in sialidase-treated DCs

The most important function of DCs is their unparalleled capacity for eliciting an adaptive immune response. This is accomplished by antigen presentation to T cells via MHC and CD1 molecules, together with co-stimulatory and a “cytokinic” signal, the latter with important consequences in skewing the T cell polarization.

Following our endocytosis/phagocytosis and maturation studies (see sections 4.1.3 and 4.2), we asked **whether the immunological function of moDCs following phagocytosis would change by removing sialic acids**. To investigate the functional consequence of the exposure of human moDCs to sialidase, the gene expression of IL-6, IL-12α, IL-1β, TNF-α and IL-10 cytokines was analyzed by quantitative real-time PCR. Although there was some sample-to-sample variation, at the end of endocytosis, **all sialidase-treated moDC samples had increased levels of IL-6 (around 2 to 50-fold) compared with the corresponding mock-treated moDCs** (Fig. 4.17). **The mRNAs for IL-12α, IL-1β and TNF-α were also augmented by the sialidase treatment** in the majority of the samples, although in one out of five samples this increase was almost insignificant (Fig. 4.17). Regarding the IL-10 expression, even though a number of moDC samples presented ± twofold increased expression, after endocytosis and sialidase treatment, two out of five samples showed a slight IL-10 down-regulation (Fig. 4.17).

As in previous assays, we also investigated the effect of sialidase itself, by analyzing the expression of these cytokines in the moDCs which had not undergone endocytosis. Our results showed that the sialidase treatment per se led to a similar increased expression of IL-12α and IL-6 (Fig. 4.17). For the IL-10, IL-1β and TNF-α, there was an up-regulation slightly higher than the sialidase-treated moDCs which had undergone endocytosis (Fig. 4.17). Therefore, **removing the sialic acid in moDCs led to an**

increased expression of IL-6, IL-1β, IL-12α and TNF-α genes. However, the range of up-regulation seemed to depend upon the individual donor and to be slightly lessened during endocytosis.

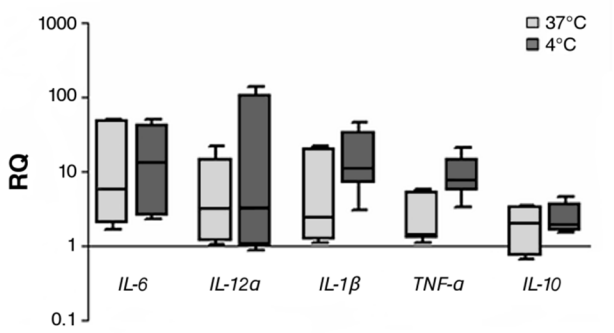


Fig. 4.17 - Expression of cytokines is altered in sialidase treated human moDCs. Sialidase treated and mock-treated (i.e., inactivated sialidase) moDCs were pulsed with ovalbumin, as described in *Materials and Methods*. After endocytosis, total RNA was used to generate cDNA that was analysed by Real Time PCR. Boxes represent minimum and maximum of the mean relative expression, **RQ**, (± SEM) of *IL-6*, *IL-12α*, *IL-1β*, *TNF-α* and *IL-10* genes. Values above or below 1 indicate, respectively, overexpression or downregulation of a specific gene after moDCs sialidase treatment, either in cells that have internalized ovalbumin (assay at 37ºC) or in the control cells (assay at 4ºC). The values represent the expression of a given gene in 5 different sialidase-treated moDCs compared with the expression in the respective mock-treated moDCs, at 4°C (dark gray) and 37°C (light gray).

Interestingly, we observed that desialylation and *E. coli* phagocytosis have an additive effect in the expression of cytokines by moDCs (Fig. 4.18A). As expected, **the expression of IL-10, IL-12, TNF-α and IL-6 increased after phagocytosis** (Fig. 4.18A) but, by adding desialylation to this condition (phagocytosis + desialylation), **moDCs expressed cytokines at their highest level** (Fig. 4.18A). This remarkable observation suggests that **removal of cell surface sialic acids, besides improving *E. coli* phagocytosis** (as previously described in section 4.2.2)**, also promotes the concomitant moDC cytokine expression**.

The Nuclear translocation Factor kappa B (NF-κB) is a key transcription factor directly associated with DC maturation (Ghosh and Hayden, 2008;Patil et al., 2010). Accordingly, we wanted to see **how the sialidase treatment might influence the activation of NF-**

κB. For that, we followed the sub-cellular distribution of p65 (RelA), a component of the NF-κB transcription complex detectable in the cytoplasm of moDCs that translocates to the nucleus upon DC maturation (May and Ghosh, 1998). Upon sialidase treatment, NF-κB nuclear translocation was limited to ~30% of the cells (Fig. 4.18B). **Following phagocytosis, nuclear translocation of NF-κB was observed in in ~80% of untreated moDCs vs. ~89% of sialidase-treated moDCs**. The percentage of moDCs with NF-κB translocated to the nucleus was comparable with the cytokine levels mentioned above, suggesting the involvement of NF-κB transcription factor in the increase of cytokine gene expression of desialylated moDCs in the tested conditions.

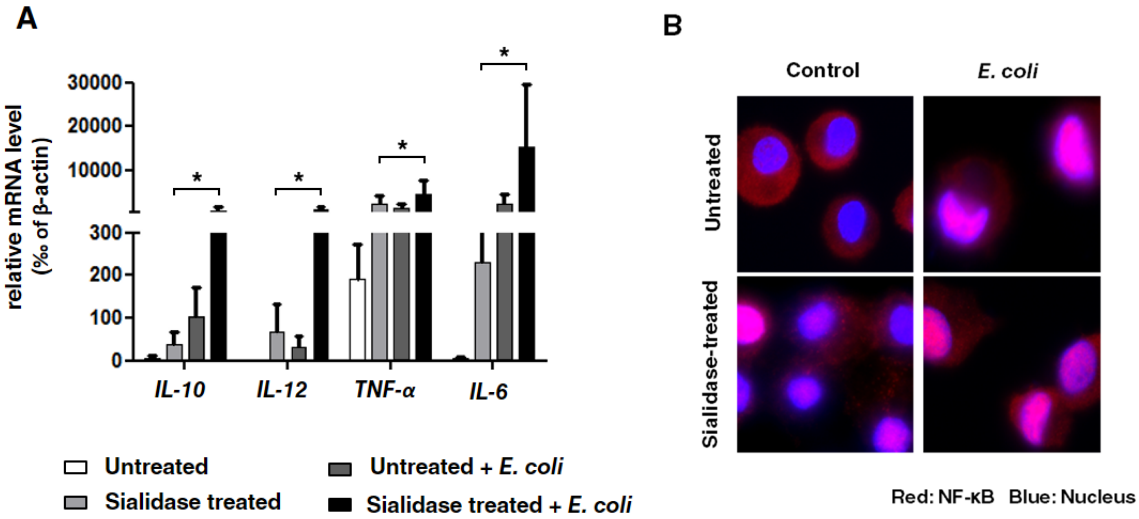


Fig. 4.18 - Sialidase treatment improves the immunological function of monocyte-derived dendritic cells (moDCs). moDCs were treated with sialidase or left untreated, and then incubated or not (control) with *Escherichia coli*. (A) The expression of IL-10, IL-12, tumor necrosis factor-α (TNF-α) and IL-6 cytokine genes was evaluated by quantitative real-time PCR in total RNA extracted moDCs (following sialidase and 1h incubation with *E. coli*). The mRNA levels of each cytokine are expressed as the permillage (‰) of the expression of the endogenous positive control, β-actin. Values represent the means of, at least, six independent assays. Statistical significance (* $p < 0.05$) refers to the difference between untreated and sialidase-treated moDCs following *E. coli* phagocytosis. (B) Representative images of the nuclear factor-κB (NF-κB) transcription factor nuclear translocation. Translocation was assessed by labelling moDCs (following sialidase and a 15-min incubation with *E. coli*) with anti-NF-B p65 (red) and staining the cell nucleus with DAPI (blue). Cells were then fixed and analyzed by combining colors through microscopy. At least 600 cells in each condition were analyzed.

4.4.2 Increased T cell priming induced by sialidase-treated human DCs

To further investigate the functional effect of sialidase treatment on human DCs, **we evaluated the ability of either sialidase-treated or mock-treated moDC to induce autologous T cell proliferation**. We have performed mixed lymphocyte cultures with moDCs loaded with tetanus toxoid (TT) from *Clostridium tetani*, treated or mock-treated with sialidase, and autologous CFSE-labelled T cells. The choice of TT as an endocytic stimulus holds up to the fact that donors had previously been immunized against this pathogen and, thus, guaranteed a strong and specific T cell response when moDCs presented TT-derived antigens. Oppositely, ovalbumin (used throughout the endocytosis studies) is a poor immunogen and, therefore, was excluded as a likely candidate for this assay. According to our results**, the number of proliferating T cells is significantly increased when antigen-loaded moDCs lose their surface sialic acid** (Fig. 4.19), as the mean value for mixed lymphocyte cultures with moDCs loaded with TT, treated or mock-treated with sialidase were 51% and 39% of $CD3^+$ cells, respectively ($p = 0.003$).

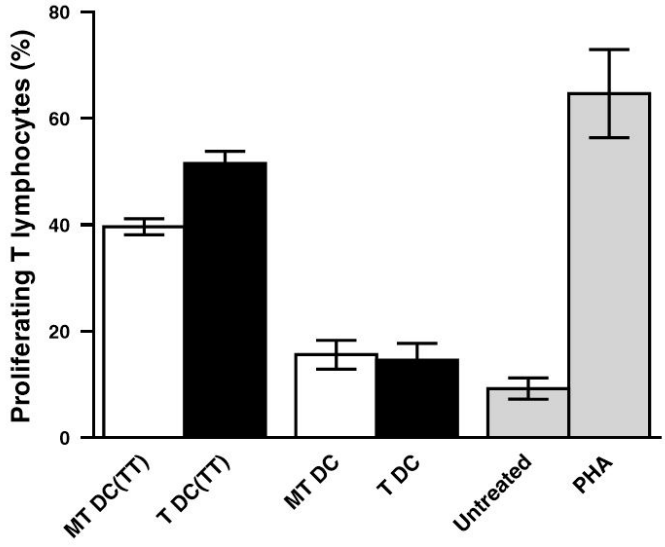


Fig. 4.19 - Increased T cell proliferation in mixed leukocyte reactions with sialidase treated DCs. Human moDCs were stimulated with TT (5 μg/ml), inactivated with mitomycin-C and treated [T DC(TT)] or mock treated (i.e., inactivated sialidase) [MT DC(TT)] with sialidase, as described in *Materials and Methods*. Control assays with unstimulated moDCs, treated (T DC) or mock treated (MT DC) with sialidase, were also performed in parallel. MoDCs were then cultured with autologous, CFSE labelled $CD14^-$ PBMCs (1:8 ratio), for 7 days. As controls (gray bars), $CD14^-$ PBMCs were cultured

alone, in the absence or presence of PHA. Cells were stained with APC-labelled anti-CD3 antibodies and analyzed by flow cytometry. Graph represents the percentage of proliferating T cells, of five individual experiments ± SEM, estimated by the percentage of CFSE diluted, $CD3^+$ cells. Significantly different values were observed for the percentage of T cell proliferation induced by T DC(TT) (* $p < 0,05$), when compared with the one induced by MT DC(TT).

To assess how desialylation in the context of phagocytosis might influence the ability of moDCs to stimulate a T cell response, **we measured IFN-γ mRNA levels in co-cultures of moDCs and autologous T cells** (Fig. 4.20C). In all controls, namely, unstimulated T cells, moDCs cultured alone (irrespective of them being challenged or not with *E. coli*) and co-cultures of T cells with untreated moDCs that did not experience phagocytosis, none or very low expressed IFN-γ mRNA was detected. However, the levels of IFN-γ mRNA expressed by T cells stimulated by moDCs experiencing *E. coli* internalization, or pre-treated with sialidase, or both, was dramatically increased. Specifically, **desialylated moDCs in response to *E. coli* exposure stimulate T cells to produce 70% more IFN-γ mRNA than fully sialylated moDCs**. These results associate well with the expected downstream events accompanying maturation, including the increased cytokine and MHC expression observed in desialylated moDCs following phagocytosis.

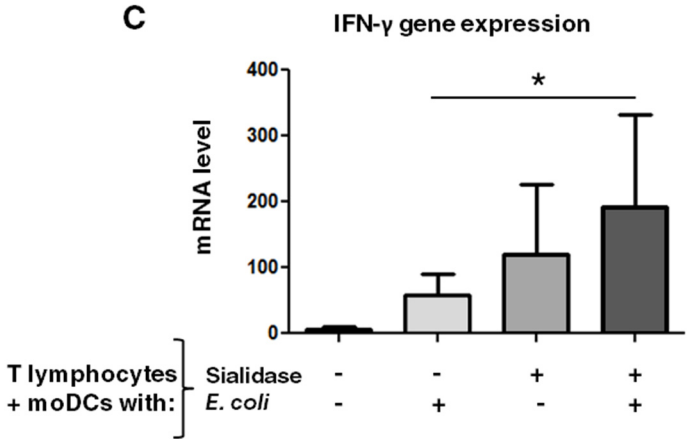


Fig. 4.20 - Sialidase treatment improves the immunological function of human moDCs. moDCs were treated with sialidase or left untreated, and then incubated or not (control) with *Escherichia coli*. (A) Interferon-γ (IFN-γ) gene expression was evaluated by quantitative real-time PCR in total RNA extracted from a 48 hr co-culture of moDCs, (following sialidase and

a 1h incubation with *E. coli*) and autologous T cells, in a DC:T cell ratio of 1:4. The IFN-γ mRNA levels are expressed as the permillage (‰) of the expression of the endogenous positive control, β-actin. Values represent the means of at least seven independent assays. Statistical significance (* $p < 0.05$) refers to the difference between untreated and sialidase-treated moDCs following *E. coli* phagocytosis.

4.4.3 Discussion

Cytokines produced by DCs are known to be important in regulating the adaptive immune response, directing it to either a pro-inflammatory immune response or to an anti-inflammatory immune response. According to our experiments investigating the functional consequences of sialic acid loss in DCs**, sialidase-mediated cleavage of sialic acid residues remarkably potentiates DC maturation and cytokine production via NF-κB translocation, resulting in an increased ability to activate T cells**. Notably, sialic acid shortage induces an up-regulation of IL-1α, IL-6 and TNF-α pro-inflammatory cytokines (Kopf et al., 1994;Dinarello, 1997) and IL-12, which drives T_h1 lymphocyte differentiation and the cell-mediated immune response (Gately et al., 1998). In contrast, the expression of IL-10, which plays a major role inducing tolerance in naïve T cells (Enk et al., 1993) and in biasing their development into T_h2 cells (Liu et al., 1998), remained basically unchanged. Increased expression of signals, such as antigen presentation and co-stimulatory molecules and IL-12, driving the development of naive precursors into T_h1 cells (Trinchieri, 1995) are key determinants for DC immunogenicity. To evaluate the cytokine profile, the expression of mRNA, but not of protein, was analyzed and it is relevant to note that a discrepancy between mRNA and protein expression is possible. Notwithstanding, our strategy is well suited to elucidate the T_h1- or T_h2-skewed DC activation profile. Our results suggest that sialidase creates an overall proinflammatory effect on moDCs, increasing their immunogenicity.

It has been reported that Gram-negative bacteria induce moDCs towards a pro-inflammatory response, with secretion of IL-12, TNF-α, IL-6 and IL-10 (Karlsson et al., 2004). In fact, here we observed that **desialylation of moDCs followed by *E. coli***

phagocytosis not only significantly improves the expression of IL-12, IL-6 and TNF-α, which are potent pro-inflammatory cytokines able to stimulate cytotoxic T cells, **but also improves the expression of IL-10**, which helps the production of B cell' immunoglobulin (Karlsson et al., 2004). This cytokine profile recalls the idea that sialidase triggers distinct intracellular signals, with relevant functional impact on the anti-pathogenic response. **We further confirm the high immunological potency of sialidase-treated moDCs following phagocytosis by demonstrating the high ability of these cells in priming T cells**. In agreement with previously produced evidence (Stamatos et al., 2004a), **the matured phenotype of moDCs, acquired through desialylation *per se*, activate the NF-κB pathway, which explains the increased cytokine gene expression**.

The idea of using sialidase treatment to alter DC immunogenicity, namely, to skew T cells towards T_h1 profile, needs further clarification. Sialidase treatment removes non-specifically α2,3-linked and α2,6-linked sialic acid from all cell surface sialylated constituents.

Mechanistically, so far, it is known that Gal-3 expression in DCs is pivotal to control the magnitude of T cell priming (Breuilh et al., 2007). There is evidence correlating reduced sialylated structures with the activation of other leucocytes such as neutrophils and T cells (Razi and Varki, 1999;Sadighi Akha et al., 2006). In the case of DCs, there is one report describing how murine DCs treated with sialidase induced specific T cell responses more efficiently (Boog et al., 1989). Interestingly, some sialyltransferases, like ST6Gal-1, are known to be down-regulated after DC maturation (see section 4.1.1) (Jenner et al., 2006), suggesting that maturation, on its own, leads to the reduction of specific sialylation.

Further functional studies are still required to further understand the competence and immune modulatory capacities of desialylated DCs. Nevertheless, because surface sialylation influences the immunogenicity of DCs upon antigen loading, our findings may

have a particular relevance to DC-based therapies and they propose that sialylation is an issue to be considered upon refinement of DC-based therapies.

Section 5

CONCLUSIONS AND FUTURE PERSPECTIVES

Dendritic cells (DCs) are important coordinators of the immune system and have a potential application as cellular vaccines in order to elicit an immune response against pathogens and tumor antigens.

Our original findings highlight that **sialic acids containing-cell surface glycans broadly influence many DC functions**. The modulation of these cells key functions – such as antigen uptake, maturation, migration and T cell stimulation – by sialic acid is reassuring towards the use of sialic acid for immunomodulation and suggesting its use as therapeutic tool.

It can be concluded from this work that **sialic acid removal enhances DC maturation while its capacity for endocytosis is compromised**. In case of phagocytosis capacity, even after the maturation process is elicited, the sialic acid-deficient DCs not only maintain their phagocytotic potential but the **loss of sialic acid is also translated by an enhanced immune potency of DCs, showed by an increase of the ability of DCs to stimulate T cells, favoring a T_h1 profile**.

In the future, it is important to clarify or to confirm the involved receptors that are affected by sialic acid. In endocytosis, Siglecs, TLRs and integrins are important candidates, of which sialic acids modification have been reported (Crocker and Varki, 2001;Feng et al., 2012;Bassaganas et al., 2014). However, it remains to be clarified whether sialic acid modifications also affect the function of these receptors in DCs. Furthermore, it is necessary to confirm whether the decreased sialylation also enhanced phagocytosis properties with other pathogens. The means to improve phagocytosis may have potential therapeutic applications in case of phagocytosis deficiency. Similar work is needed regarding T cell stimulation, where it is necessary to determine the full immunomodulating properties of desialylated DCs. The identification of the involved cell surface receptors and/or the mechanisms responsible for the observed higher ability of desialylated DCs to activate T cell response is paramount to better fine tune DC:T cell

interactions. The MHC class I and II, are important candidates, which were identified among the sialylated proteins expressed by moDCs. However, it remains to be clarified how sialic acid modifications affect the antigen presentation process. Also to be determined is the sialic acid-driven involvement of receptors involved in the formation of the immunological cleft (i.e., the contact area between DCs and T cells) such as galectins and integrins or even if the classic maturation markers are functionally altered when asialylated. These mechanisms may result from the absence of sialylation, at the moment of antigen presentation, but may also result from the same mechanisms that upregulated pro-inflammatory cytokine expression.

It was already established that sialic acid is a crucial component in DC adhesion. Nevertheless, this importance was limited to selectin ligands, and in particular to O-glycans containing α2,3-bound sialic acid, sLe^X in PSGL-1 protein (Silva et al., 2011). Here, we added information to that body of evidence by clearly demonstrating that **DC homing also depends on α2,6-sialylated N-glycans**.

The homing/migration question is the most prominent issue, concerning DC-based therapies. In spite of successful patient-derived DCs ability to elicit the desired adaptive response, these functions are compromised by the weak ability of DCs to migrate to lymph nodes. Knowing that a considerable part of the adhesion processes depends on glycosylated proteins, where the glycan moiety is functionally crucial, we expect that a better understanding of the α2,6-sialic acid-mediated migration can contribute towards the establishment of effective DC-based immunotherapies.

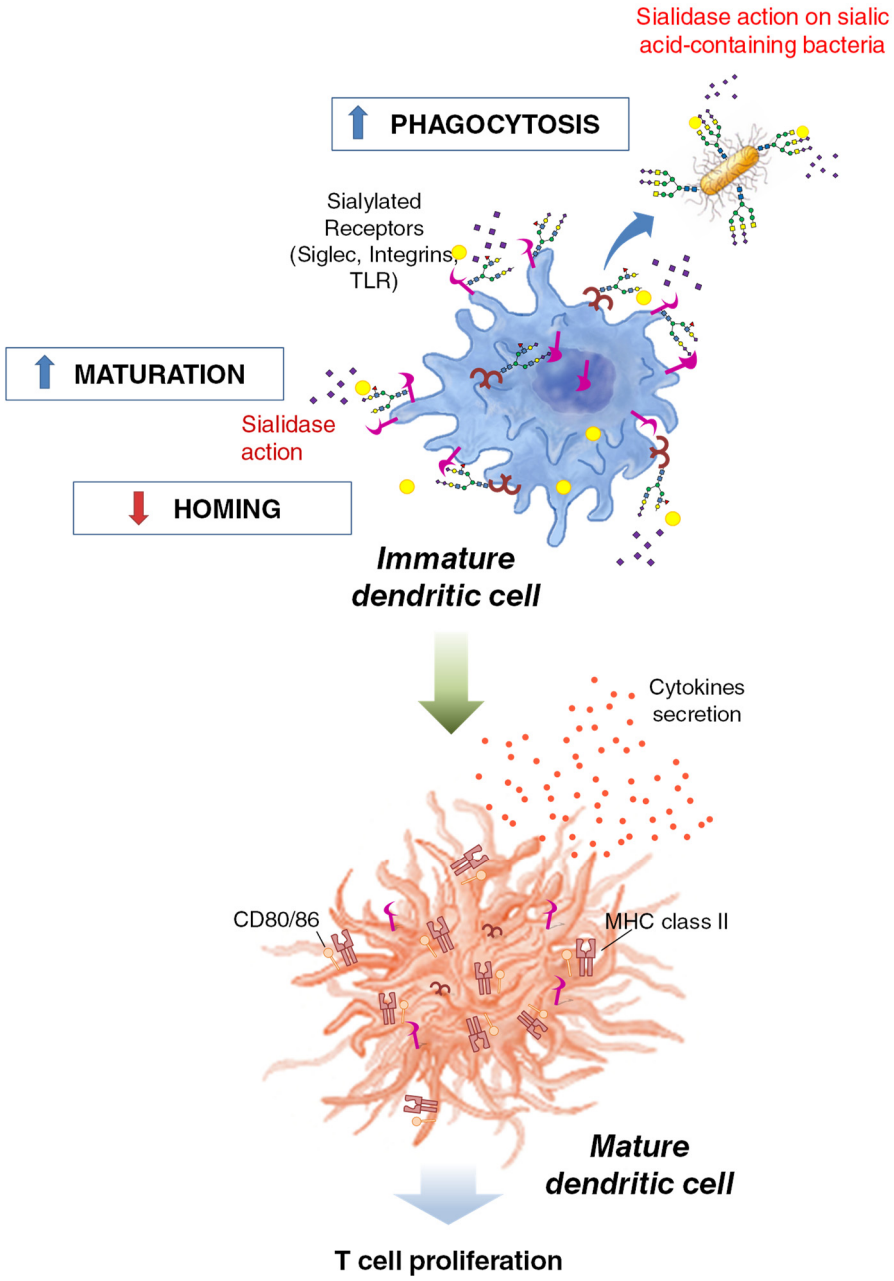


Fig. 6.1 – Overall impact of sialic acid on principal DC functions.

The study of intracellular signaling pathways has suggested that **the ERK 1/2 MAPK signalling pathway is activated upon sialic acid removal**. Activation of this pathway may explain the altered functions observed in desialylated DCs, such as altered cytokine expression. Besides, this pathway is known to be activated by integrins, as well as other receptors. Thus, considering our analysis of sialylated DC receptors, one can suggest **the β_2 integrin family as a likely candidate to be sialylated in human moDCs**. β_2

integrins are important for DC migration and therefore this could explain the observed reduction of homing of desialylated DCs. Nevertheless, further investigations are still necessary to be performed and other molecular effectors should not be discarded.

Probing for α2,6-sialylated N-glycan molecules in the ECM and lymphatic tissues can provide, similar to the invaluable contribution of mass spectrometry for our results with DCs, new leads to clarify the intervenients. Moreover, other secreted molecules, such as the previously referred galectins, should also be functionally studied as potential modulators of DC homing, in particular its expression by – and functional impact on – sialidase-treated or α2,6-sialic acid deficient DCs.

At this stage, our understanding of the underlying mechanisms linking a certain sialic acid structure and function is still limited and the interpretation of the available data assumes a considerable difficulty when evoking the diversity of receptors and the multiple signaling pathways used by DCs. The original work here presented can be a significant contribution in the growing field of glyco-immunology, especially regarding DCs, where no clear piece of evidence relating DC function and sialylation was available. However, the information regarding the sialic acid content of several receptors known to mediate functions such as endocytosis, migration and T cell priming is limited and hinders the development of important tools to manipulate DCs and to guarantee its applicability from bench to bedside. The question of whether the modulation of DC's sialic acid content can improve DC-base immunotherapy, by dictating the type of response that is triggered by DCs, remains open for now.

In the future, should the open questions be answered, solid ground for applications in DC immunotherapy can be attained. Understanding the binomial endocytosis-maturation on a perspective of Glycobiology, and studying it in the context of infection, we could unleash the mechanism of DC's antigen presenting capabilities, thus providing powerful, tailor-made new therapeutic tools. The full determination of the proteins whose glycans are sialylated and understanding the role of such modification would unravel many

therapeutic targets, and understanding how these targets may modulate the many aspects of DC functionality is relevant for immunomodulation purposes. For instance, using *in vitro* and *in vivo* models and mapping the glycome in adhesion molecules and glycome-mediated signalling potential, could become the turning point to already existing forms of therapy that still find its 'Achilles heel' in adhesion. Modulating all these processes, intracellularly – and not just using exogenous, coarser methods –, by performing true Glycoengineering is, thus, the way to which we hope to have contributed with this work.

Section 6

ANNEXES

7.1 Annex I – Results of MALDI TOF/TOF determination of N-α2,6-sialylated proteins, in human moDCs.

Entry name	Protein name	Mw (Da)
ITAM_HUMAN	Integrin alpha-M	127179
MA2B1_HUMAN	Lysosomal alpha-mannosidase	113744
ECE2_HUMAN	Endothelin-converting enzyme	99773
GELS_HUMAN	Gelsolin	85698
ITB2_HUMAN	Integrin beta-2	84782
PERM_HUMAN	Myeloperoxidase	83869
CD44_HUMAN	CD44 antigen	81538
TRFL_HUMAN	Lactotransferrin	78182
BGAL_HUMAN	Beta-galactosidase	76075
KNG1_HUMAN	Kininogen-1	71957
EST1_HUMAN	Liver carboxylesterase 1	62521
DHE4_HUMAN	Glutamate dehydrogenase 2, mitochondrial	61434
DHE3_HUMAN	Glutamate dehydrogenase 1, mitochondrial	61398
TPP1_HUMAN	Tripeptidyl-peptidase 1	61248
STK39_HUMAN	STE20/SPS1-related proline-alanine-rich protein kinase	59474
OXSR1_HUMAN	Serine/threonine-protein kinase OSR1	58022
KPYM_HUMAN	Pyruvate kinase isozymes M1/M2	57937
ATPB_HUMAN	ATP synthase subunit beta, mitochondrial	56560
VATB2_HUMAN	V-type proton ATPase subunit B, brain isoform	56501
PCP_HUMAN	Lysosomal Pro-X carboxypeptidase	55800
6PGD_HUMAN	6-phosphogluconate dehydrogenase, decarboxylating	53140
CISY_HUMAN	Citrate synthase, mitochondrial	51713
IDHP_HUMAN	Isocitrate dehydrogenase [NADP], mitochondrial	50909
GDIB_HUMAN	Rab GDP dissociation inhibitor beta	50663
GDIA_HUMAN	Rab GDP dissociation inhibitor alpha	50583
CBPM_HUMAN	Carboxypeptidase M	50514
EFTU_HUMAN	Elongation factor Tu, mitochondrial	49542
CALR_HUMAN	Calreticulin	48142
IF4A3_HUMAN	Eukaryotic initiation factor 4A-III	46871
IDHC_HUMAN	Isocitrate dehydrogenase [NADP] cytoplasmic	46656
IF4A2_HUMAN	Eukaryotic initiation factor 4A-II	46402
IF4A1_HUMAN	Eukaryotic initiation factor 4A-I	46154
PGK2_HUMAN	Phosphoglycerate kinase 2	44796
PGK1_HUMAN	Phosphoglycerate kinase 1	44615
IGHG3_HUMAN	Ig gamma-3 chain C region	41287
1C17_HUMAN	HLA class I histocompatibility antigen, Cw-17 alpha chain	41238
1A25_HUMAN	HLA class I histocompatibility antigen, A-25 alpha chain	41218
1A26_HUMAN	HLA class I histocompatibility antigen, A-25 alpha chain	41218

1C02_HUMAN	HLA class I histocompatibility antigen, Cw-2 alpha chain	41095
1A66_HUMAN	HLA class I histocompatibility antigen, A-66 alpha chain	41082
1A34_HUMAN	HLA class I histocompatibility antigen, A-34 alpha chain	41055
1A32_HUMAN	HLA class I histocompatibility antigen, A-32 alpha chain	41048
1A43_HUMAN	HLA class I histocompatibility antigen, A-43 alpha chain	41033
1A31_HUMAN	HLA class I histocompatibility antigen, A-31 alpha chain	41004
1C04_HUMAN	HLA class I histocompatibility antigen, Cw-4 alpha chain	40995
1A69_HUMAN	HLA class I histocompatibility antigen, A-69 alpha chain	40977
1C06_HUMAN	HLA class I histocompatibility antigen, Cw-6 alpha chain	40969
1C01_HUMAN	HLA class I histocompatibility antigen, Cw-1 alpha chain	40965
1A11_HUMAN	HLA class I histocompatibility antigen, A-11 alpha chain	40937
1A36_HUMAN	HLA class I histocompatibility antigen, A-36 alpha chain	40934
1C18_HUMAN	HLA class I histocompatibility antigen, Cw-18 alpha chain	40933
1A02_HUMAN	HLA class I histocompatibility antigen, A-2 alpha chain	40922
1C05_HUMAN	HLA class I histocompatibility antigen, Cw-5 alpha chain	40912
1A68_HUMAN	HLA class I histocompatibility antigen, A-68 alpha chain	40909
1A30_HUMAN	HLA class I histocompatibility antigen, A-30 alpha chain	40905
1A33_HUMAN	HLA class I histocompatibility antigen, A-33 alpha chain	40892
HLAH_HUMAN	Putative HLA class I histocompatibility antigen, alpha chain H	40892
1A74_HUMAN	HLA class I histocompatibility antigen, A-74 alpha chain	40891
1C12_HUMAN	HLA class I histocompatibility antigen, Cw-12 alpha chain	40886
1C15_HUMAN	HLA class I histocompatibility antigen, Cw-15 alpha chain	40863
1A29_HUMAN	HLA class I histocompatibility antigen, A-29 alpha chain	40863
1C03_HUMAN	HLA class I histocompatibility antigen, Cw-3 alpha chain	40861
1A01_HUMAN	HLA class I histocompatibility antigen, A-1 alpha chain	40846
1A03_HUMAN	HLA class I histocompatibility antigen, A-3 alpha chain	40841
1C14_HUMAN	HLA class I histocompatibility antigen, Cw-14 alpha chain	40838
1A80_HUMAN	HLA class I histocompatibility antigen, A-80 alpha chain	40792
1C08_HUMAN	HLA class I histocompatibility antigen, Cw-8 alpha chain	40773
1C16_HUMAN	HLA class I histocompatibility antigen, Cw-16 alpha chain	40753
1A23_HUMAN	HLA class I histocompatibility antigen, A-23 alpha chain	40733
1A24_HUMAN	HLA class I histocompatibility antigen, A-24 alpha chain	40689
1C07_HUMAN	HLA class I histocompatibility antigen, Cw-7 alpha chain	40649
1B59_HUMAN	HLA class I histocompatibility antigen, B-59 alpha chain	40584
1B49_HUMAN	HLA class I histocompatibility antigen, B-49 alpha chain	40581
1B47_HUMAN	HLA class I histocompatibility antigen, B-47 alpha chain	40571
1B51_HUMAN	HLA class I histocompatibility antigen, B-51 alpha chain	40566
1B50_HUMAN	HLA class I histocompatibility antigen, B-50 alpha chain	40541
1B41_HUMAN	HLA class I histocompatibility antigen, B-41 alpha chain	40539
1B52_HUMAN	HLA class I histocompatibility antigen, B-52 alpha chain	40521
1B40_HUMAN	HLA class I histocompatibility antigen, B-40 alpha chain	40505
1B55_HUMAN	HLA class I histocompatibility antigen, B-55 alpha chain	40496
1B53_HUMAN	HLA class I histocompatibility antigen, B-53 alpha chain	40495

1B44_HUMAN	HLA class I histocompatibility antigen, B-44 alpha chain	40481
1B56_HUMAN	HLA class I histocompatibility antigen, B-56 alpha chain	40478
1B78_HUMAN	HLA class I histocompatibility antigen, B-78 alpha chain	40478
1B13_HUMAN	HLA class I histocompatibility antigen, B-13 alpha chain	40474
1B07_HUMAN	HLA class I histocompatibility antigen, B-7 alpha chain	40460
1B37_HUMAN	HLA class I histocompatibility antigen, B-37 alpha chain	40456
1B35_HUMAN	HLA class I histocompatibility antigen, B-35 alpha chain	40455
1B46_HUMAN	HLA class I histocompatibility antigen, B-46 alpha chain	40440
1B73_HUMAN	HLA class I histocompatibility antigen, B-73 alpha chain	40435
1B27_HUMAN	HLA class I histocompatibility antigen, B-27 alpha chain	40428
1B82_HUMAN	HLA class I histocompatibility antigen, B-82 alpha chain	40421
1B38_HUMAN	HLA class I histocompatibility antigen, B-38 alpha chain	40416
1B45_HUMAN	HLA class I histocompatibility antigen, B-45 alpha chain	40414
1B81_HUMAN	HLA class I histocompatibility antigen, B-81 alpha chain	40400
1B15_HUMAN	HLA class I histocompatibility antigen, B-15 alpha chain	40388
1B54_HUMAN	HLA class I histocompatibility antigen, B-54 alpha chain	40380
1B48_HUMAN	HLA class I histocompatibility antigen, B-48 alpha chain	40362
1B14_HUMAN	HLA class I histocompatibility antigen, B-14 alpha chain	40358
1B67_HUMAN	HLA class I histocompatibility antigen, B-67 alpha chain	40342
1B58_HUMAN	HLA class I histocompatibility antigen, B-58 alpha chain	40337
1B42_HUMAN	HLA class I histocompatibility antigen, B-42 alpha chain	40333
1B08_HUMAN	HLA class I histocompatibility antigen, B-8 alpha chain	40331
1B39_HUMAN	HLA class I histocompatibility antigen	40328
1B18_HUMAN	HLA class I histocompatibility antigen, B-18 alpha chain	40275
1B57_HUMAN	HLA class I histocompatibility antigen, B-57 alpha chain	40224
HLAE_HUMAN	HLA class I histocompatibility antigen, alpha chain E	40157
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	39420
HLAF_HUMAN	HLA class I histocompatibility antigen, alpha chain F	39062
CAPG_HUMAN	Macrophage-capping protein	38499
HLAG_HUMAN	HLA class I histocompatibility antigen, alpha chain G	38224
CD1C_HUMAN	T-cell surface glycoprotein CD1c	37654
LSP1_HUMAN	Lymphocyte-specific protein 1	37192
CD1A_HUMAN	T-cell surface glycoprotein CD1a	37077
CD1B_HUMAN	T-cell surface glycoprotein CD1b	36939
IGHG2_HUMAN	Ig gamma-2 chain C region	36901
IGHG1_HUMAN	Ig gamma-1 chain C region	36106
MPRD_HUMAN	Cation-dependent mannose-6-phosphate receptor	30993
2B1B_HUMAN	HLA class II histocompatibility antigen, DRB1-11 beta chain	30160
2B14_HUMAN	HLA class II histocompatibility antigen, DRB1-4 beta chain	30112
DRB5_HUMAN	HLA class II histocompatibility antigen, DR beta 5 chain	30056
2B1G_HUMAN	HLA class II histocompatibility antigen, DRB1-16 beta chain	30030
2B18_HUMAN	HLA class II histocompatibility antigen, DRB1-8 beta chain	30004
2B1A_HUMAN	HLA class II histocompatibility antigen, DRB1-10 beta chain	30002
DRB3_HUMAN	HLA class II histocompatibility antigen, DR beta 3 chain	29962

2B11_HUMAN	HLA class II histocompatibility antigen, DRB1-1 beta chain	29914
2B19_HUMAN	HLA class II histocompatibility antigen, DRB1-9 beta chain	29826
2B17_HUMAN	HLA class II histocompatibility antigen, DRB1-7 beta chain	29822

Section 7

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