ELUCIDATING THE ROLE OF GLYCANS IN LEUCOCYTE FUNCTION

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Tese para obtenção do grau de Doutor em Ciências da Vida,
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Abstract

Glycosylation is the most widely form of protein post-translational modification and is involved in many physiological and pathological processes. Specifically, certain patterns of glycosylation are associated with determined stages of cell differentiation and can modulate processes like cell-signaling and migration and host-pathogen interactions. As such, glycosylation plays a crucial role in the modulation of several immune events. However, how glycans execute this immune-modulation and, therefore, influence immunity is still poorly unknown. Specifically, some terminal sialic acid-modified determinants are known to be involved in several physiological immune processes, including leukocyte trafficking into sites of inflammation and cell immune activation. Therefore, in this work, we sought to investigate more deeply how the expression of these glycosidic structures affects events form both innate and adaptive immune responses.

To this end, we divided our work into three main parts:

1) Immunity critically depends on the ability of sentinel circulating cells to infiltrate injured sites, of which leukocyte binding to endothelial E-selectin is the critical first step. Thus, we first analyzed the structure and function of the E-selectin ligands expressed on native human peripheral blood mononuclear cells (PBMCs), providing novel insights into the molecular effectors governing adhesion of circulating monocytes, and of circulating $CD4^{+}$T, $CD8^{+}$T and B cells, to vascular endothelium under hemodynamic shear conditions. Strikingly, monocytes show a higher ability to tether and roll on endothelial cells than lymphocyte subsets. This is due to the fact that human circulating monocytes uniformly display a wide repertoire of E-selectin binding glycoproteins, namely the E-selectin-binding glycoforms of CD43 (CD43E) and CD44 (HCELL), in addition to the previously described E-selectin-binding glycoform of PSGL-1 (CLA). In addition, we also observed a differential ability of the different lymphocyte subsets to bind to E-selectin under hemodynamic shear stress conditions, and these differences were highly correlated with their individual expression of E-selectin binding glycoproteins. While $CD4^{+}$T cells show a robust E-selectin binding ability,

$CD8^+$T and B cells show little to no E-selectin reactivity. $CD4^+$T cell potent E-selectin rolling activity is conferred by HCELL expression, in addition to the previously reported E-selectin-binding glycoproteins CD43E and CLA. $CD8^+$T cells display no HCELL and low amounts of CLA and CD43E, whereas B cells lack E-selectin ligand expression. Moreover, enforced exofucosylation of cell surface of these cells noticeably increases expression of functional E-selectin ligands among all leukocytes subsets, with cell type-dependent specificity in the protein scaffolds that are modified. Taken together, these findings redefine our understanding of the molecular mechanisms governing the trafficking patterns of PBMCs that are relevant in the context of acute or chronic inflammatory conditions.

2) The ability of circulating dendritic cells (DCs) to extravasate at inflammatory sites is critical to the success of DC-based therapies. Therefore, we assessed the structure and function of adhesion molecules mediating the transendothelial migration (TEM) of human monocyte derived-DCs (mo-DCs), obtained either by CD14 positive immune-magnetic selection (CD14-S) or by plastic adherence of blood monocytes (PA-S). We report for the first time that the E-selectin binding glycoforms of PSGL-1, CD43 and CD44 are all expressed on CD14-S mo-DCs, in contrast to PA-S mo-DCs that express only CLA. Importantly, CD44 engagement on CD14-S mo-DCs, but not on PA-S mo-DCs, triggers VLA-4-dependent adhesiveness and programs TEM in absence of chemokine gradient. We also analyzed the E-selectin ligands expressed on mo-DCs generated from umbilical cord blood (UCB) monocytes, and unexpectedly, UCB mo-DCs do not express any glycoprotein with E-selectin reactivity. Furthermore, exoglycosylation of human mo-DCs using an α(1,3)-fucosyltransferase significantly increases expression of HCELL, and therefore exofucosylated mo-DCs exhibit an augmented ability to bind to E-selectin under hemodynamic shear stress conditions. These findings highlight a role for HCELL engagement in priming TEM of CD14-S mo-DCs, and suggest that strategies to enforce HCELL expression could boost mo-DC recruitment to inflammatory sites.

3) Another obstacle to achieve the promising success of DC-based vaccines is the establishment of efficient approaches that could successfully enhance maturation and cross-presentation ability of DCs. Therefore, we investigated an alternative approach that can overcome this problem. Through removal of sialic acid content from DC cell surface we are able to elicit maturation of both human and mouse DCs. Notably, desialylated human and murine DCs showed enhanced ability to induce autologous T cell to proliferate, to secrete Th1 cytokines and to kill tumor cells. Moreover, desialylated DCs display enhanced cross-presentation of tumor antigens to cytotoxic $CD8^+$ T cells. Collectively, this study offers key insight to optimize the ability of DCs to boost anti-tumor immune responses, and indicates that the treatment with an exogenous sialidase is a powerful new technology to improve the efficacy and applicability of DC-based vaccines.

Overall, our findings show how glycosylation and its manipulation can modulate immunity. Concretely, through an exofucosylation reaction we are able to greatly augment the ability of leukocytes to extravasate into injured tissues, while removal of sialic acid moieties from cell surface of DCs, significantly potentiate their ability to induce anti-tumor cytotoxic T cell-mediate responses.

Resumo

O processo de glicosilação é a modificação pós-traducional de proteínas mais comum e está envolvido em vários processos fisiológicos e patológicos. Especificamente, certos perfis glicosídeos estão correlacionados a estados específicos de diferenciação celular, e podem modular vários eventos celulares, como sinalização celular, migração celular e interações hospedeiro-patogénio. Assim sendo, a glicosilação desempenha um papel crucial na modulação de vários processos imunológicos. No entanto, permanece por esclarecer como as estruturas glicosídicas influenciam a imunidade. Especificamente, algumas estruturas glicosídicas terminais que estão modificadas pela ligação de ácido siálico desempenham um papel importante em várias funções do sistema imune, nomeadamente migração leucocitária em contexto de inflamação e ativação de células imunes.

Como tal, este trabalho teve como objectivo investigar como a expressão de certos glicanos influencia componentes importantes da resposta imune inata e adaptativa.

Este trabalho está dividido em três componentes principais:

1) A imunidade está amplamente dependente da habilidade das células circulantes migrarem para os tecidos inflamados, sendo que a ligação de leucócitos à E-selectina endotelial é o primeiro passo. Assim, nós analisámos a estrutura e função dos ligandos de E-selectina que são expressos pelas células humanas mononucleares de sangue periférico (PBMCs), fornecendo novos conhecimentos para a compreensão dos intervenientes moleculares que mediam a ligação dos monócitos, células $CD4^+$ e $CD8^+$T e células B ao endotélio vascular. Surpreendentemente, os monócitos apresentaram maior capacidade de ligação à E-selectina comparativamente aos linfócitos. Esta observação pode ser explicada pelo facto de os monócitos humanos expressarem, uniformemente, um vasto reportório de glicoproteínas que exibem afinidade de ligação à E-selectina, nomeadamente: as glicoformas do CD43 (CD43E) e do CD44 (HCELL), em adição à já previamente reportada glicoforma da PSGL-1 (CLA).

Consistentemente, a diferente capacidade que as diversas populações linfocitárias apresentam de se ligar à E-selectina, está integralmente relacionada com a sua expressão de glicoproteínas com afinidade de ligação à E-selectina. Enquanto que as células $CD4^+$T apresentam uma elevada reatividade à E-selectina, as células $CD8^+$T e B demonstram pouca ou nenhuma capacidade de ligação à E-selectina. Esta atividade de ligação à E-selectina das células $CD4^+$T é conferida pela expressão de HCELL, em adição às já previamente reportadas CLA e CD43E. As células $CD8^+$ T não expressam HCELL e apenas expressam pequenas quantidades de CLA e CD43E, enquanto que as células B não expressam ligandos de E-selectina. Mais, a exofucosilação da superfície destas células, levou ao dramático aumento da expressão dos ligandos de E-selectina em todos as populações leucocitárias, verificando-se que a criação de certos ligandos de E-selectina está dependente do tipo de célula, após fucosilação. Colectivamente, estes resultados redefinem o nosso conhecimento acerca dos mecanismos moleculares que governam o tráfico das células mononucleares de sangue periférico em contexto de inflamação.

2) A habilidade das células dendríticas (DCs) para extravasarem em locais de inflamação é crucial para o sucesso da terapia com DCs. Assim, analisámos a estrutura e função das moléculas de adesão que mediam a migração transendotelial (TEM) das DCs. Para isso, foram usadas DCs geradas a partir da diferenciação de monócitos (mo-DCS), obtidos quer pelo métodos de separação imuno-magnética de células $CD14^+$ (CD14-S) ou por isolamento por aderência ao plástico (PA-S). Os resultados obtidos indicam que as glicoformas de ligação à E-selectina de PSGL-1, CD43 e CD44 são expressas pelas CD14-S mo-DCs, enquanto que as PA-S mo-DCs expressam apenas CLA. É importante notar que a ligação do CD44 nas mo-DCs, mas não nas PA-S mo-DCs, desencadeia a ativação e consequente adesão da VLA-4 ao endotélio na ausência de um gradiente de quimiocinas. Procedeu-se também à análise dos ligandos E-selectina expressos em mo-DCs geradas a partir de monócitos do sangue do cordão umbilical (UCB) e, inesperadamente, as UCB mo-DCs não expressam qualquer

glicoproteína com reatividade à E-selectina. Além disso, a exofucosilação das mo-DCs humanas utilizando uma α(1,3)-fucosiltransferase aumenta significativamente a expressão de HCELL e, portanto, estas células apresentam uma capacidade aumentada para se ligarem à E-selectina em condições de fluxo hemodinâmico. Estes resultados destacam o papel do HCELL no desencadeamento do TEM das CD14-S mo-DCs e sugerem que estratégias para potenciar a expressão de HCELL poderão impulsionar o recrutamento de mo-DCs para locais de inflamação.

3) Outro obstáculo para alcançar o sucesso promissor de vacinas baseadas em DCs é o estabelecimento de abordagens eficientes que poderão melhorar o estado de maturação e apresentação antigénica das DCs. Por conseguinte, foram investigadas abordagens alternativas que podem superar este obstáculo. Através da remoção de ácido siálico de superfície celular das DCs, conseguiu-se induzir a maturação de DC humanas e de ratinhos. Notavelmente, tanto as DCs humanas como as de ratinho, ao serem desialiladas mostraram uma capacidade aumentada para induzir a proliferação de células T, para secretar citocinas Th1 e para induzir a morte específica de células tumorais. Em adição, as DCs desialiladas apresentam uma maior capacidade de apresentação cruzada de antigénios tumorais às células T citotóxicas. Colectivamente, o presente estudo oferece uma visão chave para optimizar a capacidade das DCs em induzir respostas imunitárias anti-tumorais, e indica que o tratamento com sialidase é uma nova tecnologia para melhorar a eficácia e aplicabilidade das vacinas baseadas em DCs.

Coletivamente, os nossos resultados demostram como a glicosilação e a sua manipulação podem modular a imunidade. Concretamente, através de uma reação de exofucosilação conseguimos aumentar fortemente a capacidade de os leucócitos extravasarem para os tecidos afectados, enquanto que a remoção dos níveis de ácido siálico da superfície celular das DCs, induz potentes respostas anti-tumorais mediadas por células T citotóxicas.

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List of Abbreviations

A

7-AAD – 7-amino-actinomycin D

APB – Adult peripheral blood

APCs – Antigen-presenting cells

APC – Allophycocyanin (fluorochrome)

Asn – Asparagine

ATF-2 – Activating transcription factor-2

B

BCR - B cell Receptor

BDCA – Blood Dendritic Cell Antigen

BMDCs – Bone Marrow-derived Dendritic Cells

BSA – Bovine serum Albumin

Bsg – Basigin

C

CCR – Chemokine (C-C motif) receptor

CD – Cluster of Differentiation

CD14-S – CD14 positive immune-magnetic selection of monocytes

CD43E – E-selectin reactive glycoform of CD43

CFSE – Carboxyfluorescein succinimidyl ester

CHO-E– Chinese hamster ovary cells transfected with full-length human E-selectin cDNA

CHO-M – Mock-transfected CHO cells

CLA– Cutaneous lymphocyte antigen (E-selectin reactive Glycoform of PSGL-1)

CLP – Common lymphoid progenitor

CMP – Common myeloid-erythroid progenitor

CMP-5-NeuAc – Cytidine 5′-monophospho- *N*-acetylneuraminic acid

CR – Consensus repeats

CRD – Carbohydrate recognition domain

C2-4GnT – Core 2-4 *N*-acetylglucosaminyltransferase

D

DCs – Dendritic Cells

Dol-P – Dolichol-phosphate

E

EDTA – Ethylenediamine tetraacetic acid

EGF – Epidermal growth factor like domain

ELAM – Endothelial-leukocyte adhesion molecule

ESL-1 – E-selectin ligand-1

ER – Endoplasmatic Reticulum

F

FasL – Fas Ligand

Fc – Fragment crystallizable (of Ig)

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

FITC – Fluorescein isothiocyanate

FLT3-L – FMS-like Tyrosine Kinase 3 Ligand

Fuc – Fucose

FT- Fucosyltransferase

G

Gal – Galactose

GalT - Galactosyltransferase

GalNAc – *N*-acetylgalactosamine

GalNAcT – *N*-acetylgalactosaminyltransferase

Glc – Glucose

GlcNAc – *N*-acetylglucosamine

GlcNAcT or GnT – *N*-acetylglucosaminyltransferase

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GMP-140 – Granule membrane protein-140

GPCRs – G-protein-coupled receptors

GPI –Glycosylphosphatidylinositol

H

HA- Hyaloronic acid

HCELL- Hematopoietic cell E-/L-selectin ligand

HLA – Human Leukocyte Antigen

HSPCs – Hematopoietic Stem and Progenitor Cells

HRP – horseradish peroxidase

HUVECs – Human umbilical vein endothelial cells

I

ICAMs – Intracellular Adhesion Molecules

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

L

LacNAc – N-acetyl-lactosamine

LAM – Leukocyte adhesion molecule

LCAM– Leukocyte-cell adhesion molecule

LECAM – Leukocyte-endothelial cell adhesion molecule

$Le^{x/a}$ – Lewis X/A antigen

LFA-1 – Lymphocyte Function-associated Antigen 1

LPS – Lipopolysaccharide

M

MAA – *Maackia amurensis* agglutinin (lectin)

MALT – Mucosa-associated lymphoid tissues

Man – Mannose

mDCs – Myeloid Dendritic Cells

MgaT1- *N*-acetylglucosaminyltransferase-1 (=GlcNAcT-I)

MHC – Major Histocompatibility Complex

mo-DCs – Monocyte derived-Dendritic Cells

MPO – Myeloperoxidase

MPO-EL – E-selectin reactive glycoform of myeloperoxidase

mRNA – Messenger Ribonucleic acid

N

Neu – Neuraminidase

Neu5Ac – *N*-acetylneuraminic acid

NK – Natural killer

NF-κB –Nuclear factor kappa B

P

PADGEM – Platelet activation-dependent granule to external membrane protein

PAMPs – Pathogen-associated molecular patterns

PA-S – Plastic adherence selection of monocytes

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffer Saline

PCR – Polymerase chain reaction

pDCs – Plasmacytoid Dendritic Cells

PE – Phycoerythrin

PerCP – Peridinin-Chlorophyll protein

PGE2 – Prostaglandin E2

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

Pro – Proline

PRRs – Pattern Recognition Receptors

PSGL-1 – P-selectin glycoprotein ligand-1

O

OST – Oligosaccharyltransferase

Ova – Ovalbumin

R

ROS – Reactive oxygen species

S

sDCs– Murine splenic DCs

sT - Sialyl-T antigen

sTn – Sialyl-Tn antigen

SDS-PAGE – Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis

Ser – Serine

Sia – Sialic acid

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 – Sialyltransferases

sT – Sialyl-T antigen

sTn – Sialyl-Tn antigen

ST3Gal – β-galactoside α2,3-sialyl-transferase

ST3GalNAc – α-*N*-acetylgalactosa-minide α2,3-sialyltransferase

s6T – Sialyl-6T antigen

ST6Gal – β-galactoside α2,6-sialyltransferase

ST6GalNAc – α-*N*-acetylgalactosa-minide α2,6-sialyltransferase

T

TBS – Tris-buffer Saline

Tc - T cytotoxic (cell)

TCR – T cell Receptor

TEM – Transendothelial Migration

TGF – Tumor Growth Factor

Th – T helper (cell)

TLR – Toll-like receptors

TNF – Tumor necrose factor

Treg – Regulatory T (cell)

U

UCB – Umbilical cord blood

V

VCAMs – Vascular Cell Adhesion Molecules

VLA-4 – Very Late Activation Protein 4

Chapter 1

Introduction

1.1 The Immune System- an overview

The functional integrity of our body and its protection from environmental agents are mediated by the immune system. The immune system can be defined as a set of integrated and complex mechanisms to protect our body against invading pathogens or foreign material.[1] This is done by the constant recognition of self from non-self determinants, which allows an effective immunity to any harmful aggressor but prevents destructive immune responses to our own body.[2,3] The immune system can be divided into two inter-related and functionally defined components: the **innate immune response** and the **adaptive or acquired immune response**.[1]

1.1.1 Innate Immune Response

The **innate immune response** corresponds to the first line of host defense against infection, i.e. comprises physical and physiological barriers, innate immune cells and soluble factors that are present and ready to mobilize and combat invading microorganisms. These protection mechanisms occur immediately after infection, are not specific to the invading agents, and do not present immunological memory.[1,2]

A variety of immune cells are responsible for cellular innate immunity. These include the cells that are capable of **phagocytosis,** a process by which ingestion of particulate material occurs – neutrophils and mononuclear phagocytes (monocytes, macrophages and dendritic cells) –, the cells that are capable of cytotoxicity – **N**atural **k**iller (NK) cells and eosinophils – and the cells mostly dedicated to immune-modulation – basophils and mast cells.[2,4,5]

The cellular components of the innate immune system recognize a limited number of structures that are characteristic of microbial agents and that are not present in mammal cells. These structures are referred to as "**P**athogen-**a**ssociated **m**olecular **p**atterns" (PAMPs). These highly conserved structures are recognized and bind to receptors present in the innate immune cells, designated as "**p**attern **r**ecognition **r**eceptors" (PRR), which include the **T**oll-**l**ike **r**eceptors (TLRs). These receptors can transduce intracellular signals that will activate anti-microbial and pro-inflammatory mechanisms in innate immune cells and can also accelerate the process of microbial internalization by the phagocytes.[6-9]

The first step in the phagocytosis process is the recognition of the PAMPs by the PRRs expressed on phagocytes, followed by internalization of the pathogens into phagosomes and fusion of phagosomes with lysosomes, to form a phagolysosome. Finally, the destruction of the external agents is mediated within the phagolysosome by hydrolytic enzymes, with nitric oxide and **r**eactive **o**xygen **s**pecies (ROS) production. The phagocyte cells include monocytes, macrophages, **d**endritic **c**ells (DCs) and neutrophils.[4,10-12]

Monocytes constitute a heterogeneous peripheral blood leukocyte population, comprising approximately 5-10% of total peripheral blood leukocytes. These cells are originated from a **c**ommon **m**yeloid-erythroid **p**rogenitor (CMP) in the bone marrow and subsequently released into the peripheral blood. In case of inflammation, monocytes leave the blood stream and migrate to peripheral tissues, where depending on the local chemical environment can further differentiate into DCs or macrophages.[13,14] Monocytes have thus an essential role in anti-microbial immunity, due to their phagocytic capacity and ability to differentiate into macrophages or DCs, which are professional **a**ntigen **p**resenting **c**ells (APCs). Nevertheless, monocyte recruitment into tissue is also associated with several pathological inflammatory processes, including atherosclerosis.[15]

Macrophages and DCs exist virtually in every tissue throughout the body, where they survey for potential signs of infection/danger.[16] After pathogen phagocytosis, the foreign antigens are broken down into smaller fragments and then presented at the cell surface of the phagocytes via the **M**ajor **H**istocompatibility **C**omplex (MHC) to lymphocytes.[12] Even though both cells are able to present antigens, macrophages are less efficient compared to DCs. DCs are the most potent APCs. Moreover, after engulfing and processing antigens, DCs migrate to the lymphoid organs where they prime T cells.[17,18] In contrast, macrophages do not leave from the site of injury and their major function is to kill the pathogen, via lysosomal proteolitic activity.[13,19] In addition to the destructive capacity of invading agents, macrophages play other roles in innate immunity through the production of cytokines, in particular **t**umor **n**ecrosis **f**actor-α (TNF-α) and **i**nter**l**eukin-1 (IL-1), which promotes inflammation. Macrophages also influence the adaptive immunity via the secretion of IL-12 that affects polarization of *naïve* T cells.[1,20]

A more accurate and complete description of DC biology and functionality is described in: **chapter 1.2.1 Biology of DCs**.

Neutrophils are the first cells to arrive to the inflammation/infection sites and are the most abundant white blood cells in the peripheral blood. These cells differentiate in the bone marrow from CMP and then are released into bloodstream, where they circulate before migrate into the tissue spaces. Similar to macrophages, neutrophils are involved in immediate removal of pathogens via intracellular death mechanisms and also by the formation of neutrophil extracellular traps.[4,9] Neutrophil granules contain a range of lethal substances that destroy or inhibit expansion of several pathogens, including toxic free oxygen radicals, chloride derivates, and a wide range of hydrolytic enzymes.[21]

NK cells can be cytotoxic against autologous cells when infected by viruses or intracellular bacteria, or when they become malignant. These cells arise from a **c**ommon **l**ymphoid **p**rogenitor (CLP) within the bone marrow and account for about 15% of total lymphocyte population.[1] The cytotoxic activity of NK is mediated by cytotoxic proteins contained in granules, perforin (a pore-forming protein) and granzyme that when released induce apoptosis of target cells.[22,23] In addition, NK cells secrete several cytokines, including IL-2, **i**nter**f**ero**n**-γ (IFN-γ), TNF-β and TNF-α, which have an adjuvant role in NK cytotoxic action.[1] The **eosinophils and basophils** develop from a bone marrow CMP and are the rarest of the white blood cells in peripheral bloodstream. These cells are mainly involved in allergic and inflammatory reactions. Eosinophils are the principal innate immune cells involved in parasitic infections, secreting chemical molecules that kill these microorganisms.[1] Basophils are the cells responsible for secreting histamine, a chemical substance that elicits vasodilation and increases vascular permeability to the sites of injury/inflammation.[1,24]

The innate and adaptive immunity do not act fully independent of each other. They cooperate with each other to produce a more effective immunity. The innate immune response stimulates the establishment of the adaptive immunity and influences its nature. Thus, when inflammation is not sufficient to remove the invading agent, its perpetuity increases the number of APCs in the lymphoid tissue, inducing the activation and migration of T cells, while certain complement proteins stimulate the activation of B lymphocytes, developing, therefore an adaptive immune response (**Figure 1**).[1,2]

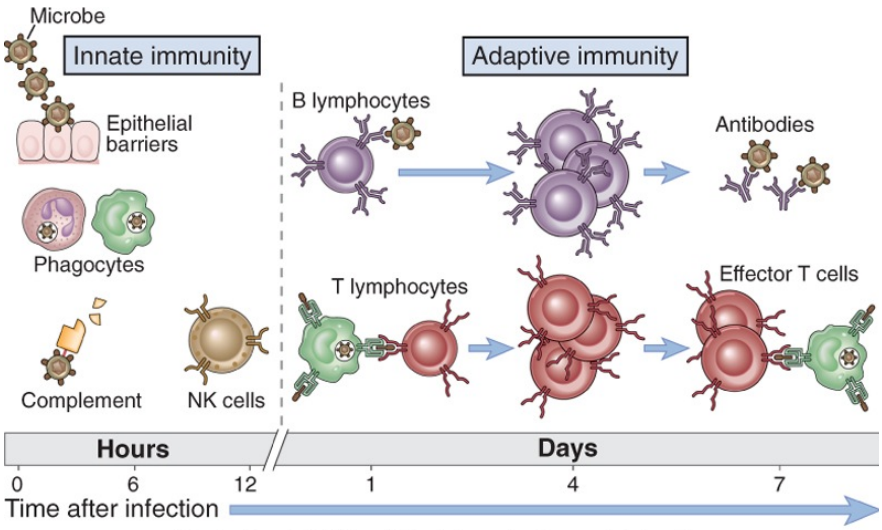

Figure 1- Integration of innate and adaptive immune response. The body has an integrated and complex defense mechanism in which several cells and tissues act in coordination to protect the host. When the innate effectors are not able to circumvent the infection/ injury, an appropriate acquired response is mounted, leading to a highly effective recognition and destruction of pathogens. *In Cellular and Molecular Immunology. 6th edition. Abbas k., et al., Philadelphia. Saunders/Elsevier.2010*

1.1.2 Adaptive Immune response

The adaptive immunity is able to specifically recognize and eliminate microorganisms and foreign molecules. Unlike the innate immune response, the adaptive response is a delayed response and presents antigen specificity, clonal proliferation of effector cells, diversity and immunological memory. There are two types of adaptive immune response: **cell-mediated immunity**, performed by T lymphocytes, and **humoral immunity**, mediated by antibodies produced by B lymphocytes.[1,25]

1.1.2.1 Cellular adaptive immunity

T cells arise from a CLP within the bone marrow, but unlike B cells, their maturation process proceeds within the thymus. Inside the thymus, T cell-precursors (also called thymocytes) undergo a set of positive and negative selection stages and as they advance throughout their development, they become mature T cells. Typically, mature T cells are tolerant to self antigens, single positive for either CD8 or CD4 glycoproteins and express a functional **T-c**ell **R**eceptor (TCR) that recognizes a specific antigen bound to the host MHC-Class I ($CD8^+$T cells) or MHC-class II ($CD4^+$T cells) molecules. Finally, mature lymphocytes egress the thymus and enter the peripheral bloodstream and recirculate between peripheral lymphoid

organs, where they search for their cognate antigen bearing-APCs. After antigen encounter, T cells become activated and undergo clonal expansion and further differentiation into effector T cells that migrate to the sites of inflammation or infection.[1,26,27]

Mature T cells can be typically categorized into two main subsets, that differ in their cell surface CD4 or CD8 expression and immunological functions: helper and cytotoxic T cells, respectively.

1.1.2.1.1 Helper T cells

Helper T cells (**Th)**, also called $CD4^+$ T cells, play a crucial role in immune system by assisting other leukocytes in their immunological roles (see **Figure 2**). Their main functions include: help B cells to differentiate into antibody-secreting cells, elicit activation, proliferation and maintenance of $CD8^+$ T cell response, regulate macrophages function and regulate/suppress immune responses in cases of auto-immunity or exacerbated immune reactions. These cells become activated after recognizing extracellular antigens that, following lysossomal-degradation, are bound to MHC-class II molecules and exposed on cell surface of APCs. Subsequently to activation, $CD4^+$ T cells are further stimulated to differentiate into effector or regulatory $CD4^+$ T cells. Depending on the type of cytokines secreted, effector $CD4^+$ T cells can be additionally subdivided into several subtypes, including Th1, Th2 and Th17.[27,28]

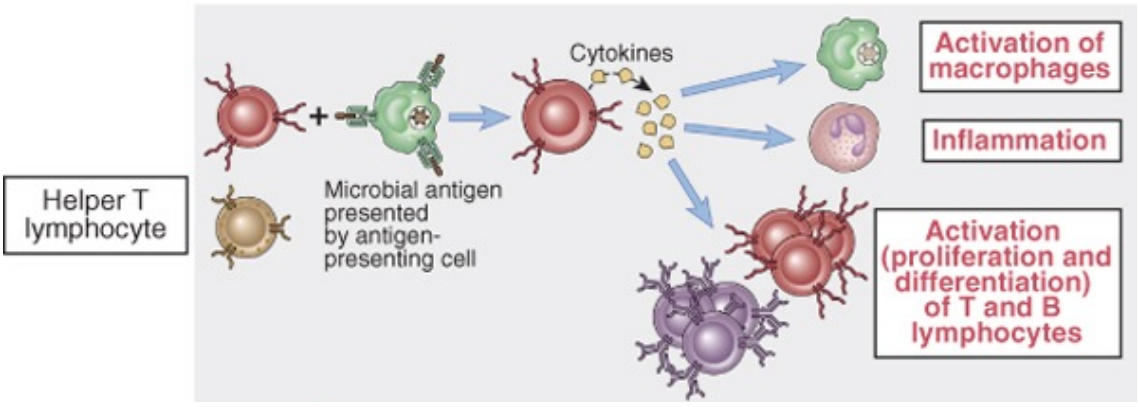

Figure 2- Role of $CD4^+$ T cells in immunity. $CD4^+$ T cells recognize, via their T cell receptors, their cognate antigens bound to MHC-Class II on APCs. Follow antigen encounter, $CD4^+$ T cells become activated, proliferate and depending on local cytokine milieu differentiate into different subsets of effector T cells. $CD4^+$ T cells orchestrate several immune responses, including help B cells to produce antibodies, enhance macrophage anti-microbicidal activity, granulocytes recruitment and activation of $CD8^+$ T cytotoxic activity. *In Cellular and Molecular Immunology. 6th edition. Abbas k., et al., Philadelphia. Saunders/Elsevier.2010*

Th1 cells are essential in promoting cellular immunity against intracellular pathogens, via activation of $CD8^+$ T cell response and phagocytes stimulation. Th1 $CD4^+$ T cells secrete high levels of IFN-γ, TNF-α and IL-2, which are pro-inflammatory cytokines. IL-12 secretion by activated DCs plays a crucial role in the *naïve* $CD4^+$ T cells polarization into Th1.[27-30]

Th2 cells are important in promoting humoral immune response, via production of antibodies; they stimulate the activation and accumulation of eosinophilis, contributing thus to host defense against helminthes, but are also involved in acute and chronic inflammation in allergic reactions. These cells produce several anti-inflammatory cytokines, including IL-4, IL-5, IL-6, IL-10 and IL-13, which tend to inhibit Th1 response. The major cytokines responsible for *naïve* $CD4^+$ T cells polarization into Th2 are IL-4 and IL-10.[27-30]

Th17 cells are critical to combat extracellular bacteria and fungi, given their ability to prompt recruitment and activation of neutrophils, via the induction of IL-8 production. Th17 cells secrete large amounts of IL-17, which has been correlated with several autoimmune and inflammatory diseases. Secretion of IL-23 and IL-1β has been involved in polarization of $CD4^+$ T into Th17.[27-31]

1.1.2.1.2 Cytotoxic T cells

Cytotoxic T cells (**Tc**), also known as $CD8^+$ T cells, provide defense against intracellular pathogens, including virus and some intracellular bacteria, and tumor cells. In contrast to $CD4^+$ T cells, $CD8^+$ T cells execute their immunological role by directly killing the infected or cancer cells, as shown in **Figure 3**. Within the lymph nodes, $CD8^+$ T cells recognize, typically, intracellular antigens that are processed and presented on the cell surface of infected or tumor cells via MHC-class I. After antigen encounter in lymphoid tissues, $CD8^+$ T cells undergo through clonal expansion and differentiate into memory or effector $CD8^+$T cells. Effector $CD8^+$ T cells migrate then to sites of infection/inflammation, where they exert their cytotoxic role.[1,25]

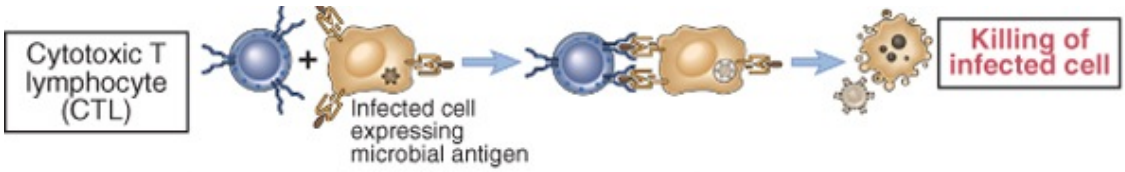

Figure 3- Effector cytotoxic T cell functions. When recognize their cognate antigen-MHC-class I complexes on the target cells, $CD8^+$ T cells become activated, proliferate and differentiate into effector T cells, which are cytotoxic to infected cells. *In Cellular and Molecular Immunology. 6th edition. Abbas k., et al., Philadelphia. Saunders/Elsevier.2010*

At sites of injury and when exposed to their target cells, effector $CD8^+$ T cells can employ different mechanisms to induce apoptosis of the target cells, as shown in **Figure 4**. Firstly, effector $CD8^+$ T cells are able to release perforin and serine proteases, including granzyme. Perforin induces transmembrane channels on plasma membrane of the target cells, allowing proteases to enter into the cytosol and, thereby, trigger caspase cascade, leading subsequently to programmed cell death of the target cell. A second mechanism to induce target cell apoptosis is through the engagement of the cell death surface receptor Fas, expressed on target cells, by Fas ligand (FasL) binding, expressed on cell surface of effector $CD8^+$T cells. Activation of Fas-FasL pathway triggers caspase cascade activation that leads to target cell apoptosis.[1,32]

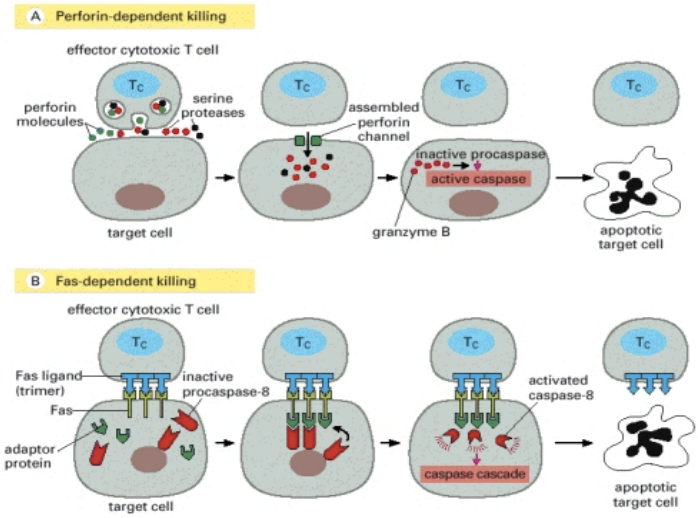

Figure 4- $CD8^+$ T cells exert their cytotoxic activity via two apoptosis-inducing mechanisms. A) When cytotoxic $CD8^+$ T cells bind to the target cells, they can release perforin molecules, which create transmembrane pores on the infected cells. This allows the entrance of granzyme into the cytosol, inducing the activation of the caspase cascade and culminating in target cell apoptosis. B) Apoptotic cell death of target cells can also be induced via engagement of cell death receptor Fas by Fas ligand binding, expressed by effector cytotoxic T cells. *In Molecular Biology of the cell. 4th edition. Alberts B, et al. New York. Garland Science.2002*

Importantly and identical to $CD4^+$ T cells, effector $CD8^+$ T cells are also prompted to differentiate into different subsets of effector cells, depending on the type of cytokines they express: Tc1 or Tc2.

Similar to Th1, **Tc1 cells** are also important in cell-mediated immunity against intracellular pathogens and are great producers of IFN-γ. Like Th1 cells, secretion of IL-12 by DCs promotes polarization of *naïve* $CD8^+$ T cells towards Tc1 phenotype.[30]

Tc2 cells are mainly committed to protect the host against helminthes and produce mostly IL-3, IL-5 and IL-13. These cells do not secrete IFN-γ and their cytotoxic activity is significant reduced compared to Tc1.[30]

Importantly and in addition to endogenous antigens, exogenous antigens can also be presented to $CD8^+$ T cells by APCs (mainly DCs) via MHC-Class I in a process called **cross-presentation**. This is how our immune system is able to induce $CD8^+$ T cell cytotoxic responses against solid tumors or virus that do not infect APCs, or whose antigens are not expressed by the target cells due to defects in the antigen presenting machinery.[33,34]

1.1.2.2 Humoral Immune Response

B cells differentiate from a CLP and when developed, immature B cells egress the bone marrow and migrate via bloodstream to spleen where they differentiate into mature *naïve* B cells. Those *naïve* mature B cells will then continuously recirculate between secondary lymphoid organs (lymph nodes, spleen and **m**ucosa-**a**ssociated **l**ymphoid **t**issues (MALT)), until they recognize an antigen. At this point, each *naïve* B cell must express a fully functional cell surface receptor (known as **B c**ell **R**eceptor (BCR)) that will bind to one specific antigen. Following the initial contact with its cognate antigen, *naïve* mature B cell will initiate clonal expansion and further differentiation into memory B cells or pre-effector B cells (plasmablasts), which, thereafter, will differentiate into antibody-secreting plasma B cells within the bone marrow or MALT. These effector plasma B cells produce and release large amounts of antibodies in the blood, which will bind to their respective antigens and promote the destruction of the pathogens via several immune-related mechanisms (**Figure 5**). The antibodies and the membrane-bound BCR will have the same antigen binding sites, i.e. the antibodies will have the same specificity to the antigen that caused initial B cell clonal expansion. In case of secondary encounter with the same antigen, peripheral blood circulating memory B cells are primed to generate a faster and more efficient antibody-mediated response.[1,35-38]

Antibodies provide immunity in four major ways: neutralization, opsonization, activation of the complement cascade and cell-induced apoptosis. Neutralizing antibodies bind to specific surface receptors on pathogens, preventing these from entering host cells. The opsonization happens when antibodies bind to invading microorganisms or antigens, and are also

recognized by phagocytes, eliciting pathogen phagocytosis. Phagocytes express antibody receptors, known as Fc receptors, which recognize and bind to the constant region (Fc) of the antibody bound to the pathogen, thus facilitating the phagocytosis process. In alternative, antibodies bound to the pathogen can activate the complement system pathway, which can either result in enhanced pathogen phagocytosis or lead to direct microbe cytolysis by the complement cascade components (C7, C8, and C9). Finally, the innate immune cells that express Fc receptors and that exert immediate cytotoxic functions (e.g., NK cells or eosinophils) can bind to the antibody-pathogen complexes and target their apoptotic activity to infected cells, tumor cells, or pathogens.[1,25,39]

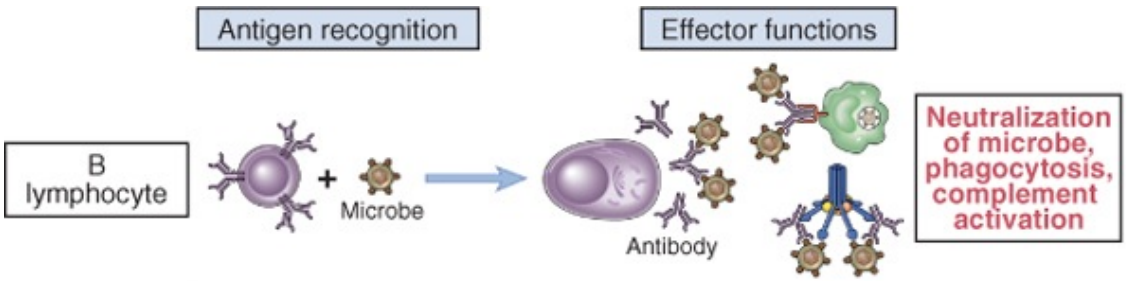

Figure 5- The humoral immune response. After encounter with its cognate antigen, the activated B cell begins to differentiate into plasma B cells and release large amounts of antibodies into the blood. *In Cellular and Molecular Immunology. 6th edition. Abbas k., et al., Philadelphia. Saunders/Elsevier.2010*

In addition to being antibody-secreting cells, B cells play also a critical role in regulating other immune mechanisms and are important links between innate and acquired immunity. After BCR engagement by antigen binding, B cells are stimulated to capture the antigen and to present it via MHC-II molecules, after antigenic degradation into smaller peptides. Therefore, B cells can also function as professional antigen-presenting cells, presenting the antigenic-peptides to $CD4^+$ T cells in a process called T-B cells cooperation.[40] Similarly to other immune cells, activated B cells (via BCR engagement) can produce cytokines, such as the pro-inflammatory cytokines IL-6 and TNF-α, playing an important role in T cell activation and expansion, as well as chemokines, regulating leukocyte recruitment.[38,41]

1.2 DC-based Immunotherapy

The concept of anti-tumor immune surveillance was first introduced by Thomas and Burnett, proposing that the immune system is able to identify tumor cells as "foreign" with regard to normal cells, and thereby trigger the effector mechanisms capable of eliminate tumor cells.[42,43] However, there are many cases where the immune system does not develop an effective response against the tumor cells.[44] Therefore, new strategies to boost, reinforce, and maintain long lasting anti-cancer immune responses are needed. DCs constitute one of the effector components of the immune system involved in the elimination of tumor cells. These cells are the antigen-presenting cells par excellence, showing a unique capacity to initiate immune response. DCs play a key role in the activation of T and B cells, as well as in the activation of NK cells and consequently in the secretion of cytokines by these effectors.[1,45,46] Moreover, DCs have the single ability to cross-present exogenous antigens to $CD8^+$ T cells via MHC-Class I. This process is crucial for the generation of cytotoxic $CD8^+$ T mediated immune response, and thereby for the generation of anti-tumor immune responses.[47] Due to their crucial role in boosting innate and adaptive immune responses, immunization of cancer patients with autologous tumor-antigen-loaded DCs has been explored in order to generate more effective and long-lasting anti-cancer immune responses. [48]

1.2.1 Biology of dendritic cells

1.2.1.1 Origin and DC subsets

DCs are a rare leukocyte population that displays high structural heterogeneity and functionality. Cellular origin, anatomical location, maturation state and T cell activation ability are some of the factors that contribute to DC heterogeneity.[49] Human DC precursors arise from a bone marrow CMP and, classically, are categorized into two major cell lineages: myeloid/conventional ($CD11c^+/CD123^-$) and lymphoid/plasmacytoid ($CD11c^-/CD123^+$).

In the blood, human myeloid DCs (mDCs) can be further divided into $CD141^+$ (BDCA-3) or $CD1c^+$ (BDCA-1) DCs and $CD14^+$ or $CD16^+$ monocyte subsets, as shown in **Figure 6**. $CD141^+$ DCs are specialized in cross-presenting antigens, via MHC class-I molecules, to $CD8^+$ T cells, thereby, eliciting $CD8^+$ T cell-mediated immune responses. $CD141^+$ DCs have also been described to polarize $CD4^+$ T cells towards a Th1 profile. Upon activation, $CD1c^+$

DCs secrete high amounts of IL-12, having an active role in the induction and expansion of IFN-γ producing $CD4^+$ and $CD8^+$ T cells, and improving anti-bacterial and antitumor immune responses.[48,50-54]

In non-lymphoid tissues, mDCs are typically distinguished by cutaneous lymphocyte antigen (CLA) and myeloid makers expression and by compartment localization. These include the $CD141^+$ DCs (CLA^+, known as Langerhans cells in skin tissue) and interstitial DCs (CLA^-, known as dermal DCs in skin tissue). The interstitial DCs can be further divided into $CD1a^+$DCs and $CD14^+$DCs. Similar to the blood $CD141^+$ DCs, non-lymphoid $CD141^+$ DCs are highly efficient in cross-presenting exogenous antigens to $CD8^+$ T cells and priming cytotoxic effector $CD8^+$ T cell differentiation. Human interstitial $CD14^+$ DCs are unique in their capacity to induce differentiation of *naïve* B cells into IgM plasma cells, thus being crucial for the development of humoral immune responses. On the other hand, interstitial $CD1a^+$DCs strongly prompt both $CD4^+$ and $CD8^+$ T cell proliferation, and favours $CD4^+$ T cell polarization towards Th2 phenotype.[53-57]

pDCs are described as the natural IFN-producing cells. This subset of DCs is characterized by CD123, BDCA-2 (CD303) and BDCA-4 expression and is found mostly in blood and lymphoid organs. pDCs also seem to play a dual role in anti-tumoral immunity. While some reports indicate that pDCs prime specific cytotoxic $CD8^+$ T and $CD4^+$ T polarization into Th1, the opposite tolerogenic role has also been observed, predominantly related with the generation of regulatory T cells (Tregs), which have an immunosuppressive activity.[48,51-54]

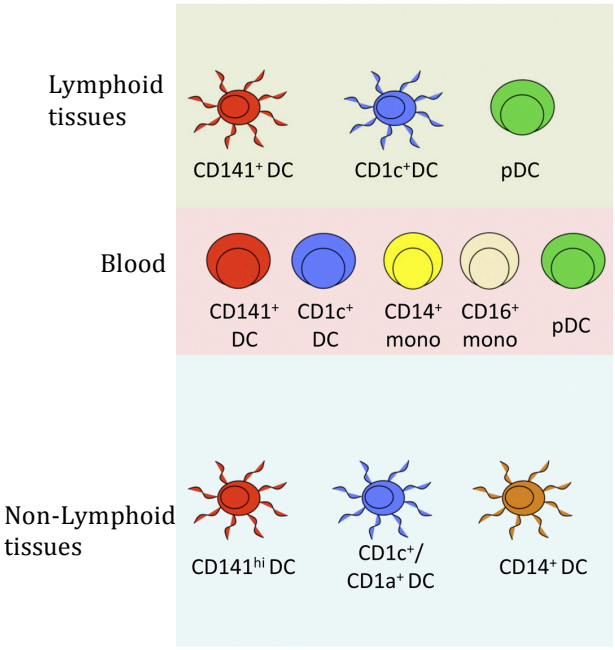

Figure 6- Subsets of human DCs. Classically, DCs are classified into two major cell lineages: myeloid (mDCs) or plasmacytoid (pDCs) DCs. pDCs are found mostly in the blood and lymphoid tissues, whereas mDCs ($CD141^+$ and $CD1c/a^+$ DCs) are found virtually in every tissue. *Adapted from Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. Haniffa M et al., Immunity. 2012*

1.2.1.2 Maturation

DCs are potentially in all tissues where they capture antigens, migrate to secondary lymphoid organs and become competent to activate *naïve* T cells. Notably, a particular characteristic of DCs is to comprise a process of differentiation depending on their local chemical milieu. In brief, DC precursors circulate in peripheral blood and subsequently penetrate into peripheral tissues, where they originate immature DCs. In their immature state, DCs are characterized by a high phagocytic and endocytic capacity and a low expression of MHC and co-stimulatory molecules. Yet, after antigen recognition, capture and processing, immature DCs differentiate into mature DCs, characterized by a reduced endocytic capacity, high expression of antigenic peptide-MHC complexes and co-stimulatory molecules (CD40, CD80 and CD86), secretion of pro-inflammatory cytokines, such as IL-12, and an increased expression of the C-C chemokine receptor type 7 (CCR7) molecule (**Figure 7**). After differentiation, mature DCs migrate to lymphoid tissues and contact with T and B cells, initiating primary immune response.[56,58]

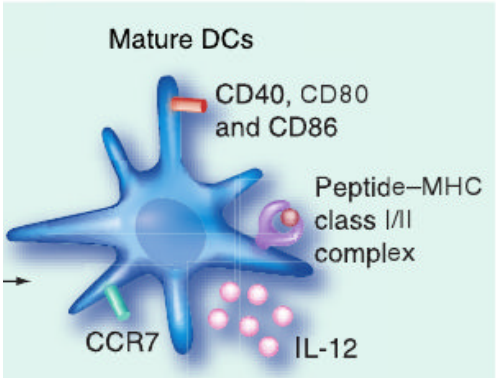

Figure 7- Schematic representation of a mature DC. After antigen phagocytosis, DCs increase expression of maturation markers, such as antigen-MHC complexes and the co-stimulatory molecules CD40, CD80 and CD86. Increased secretion of the Th1-inducing cytokine IL-12 and expression of CCR7, critical for DC migration to lymph nodes, are also features of mature DCs. *Adapted from Directing dendritic cell immunotherapy towards successful cancer treatment. Sabado RL and Bhardwaj N. Immunotherapy. 2010.*

1.2.1.3 DCs and T cell activation

Upon arrival to the lymph nodes, mature DCs initiate the contacts with T cells via three cooperative signals. Firstly, through their MHC molecules, DCs bind to TCR expressed on cell surface of *naïve* T cells. The second signal comes from the interaction of B7 co-stimulatory molecules, such as CD80 and CD86, on the surface of DCs with the CD28 co-stimulatory receptor on the T cell surface.[48,59] Finally, the third signal results from cytokine secretion by DCs and will define which type of Th or Tc response will occur. As mentioned previously, secretion of IL-12 is crucial for the induction of Th1 and Tc1 responses.[60] On the other hand, IL-4 and IL-10 are implicated in Th2 and Tc2 immune responses, whereas IL-23 and IL-1β promotes differentiation of T cells into Th17. Secretion of IL-10 by activated DCs is also highly correlated with tolerance inducement via polarization of *naïve* Th cells into Tregs.[28,61-63]

1.2.2 DCs in tumor microenvironment

Many studies have demonstrated that a defect in DC functionality is a key factor that may lead to immune escape of tumors. The failure of DC activity is often caused by factors secreted by tumors that possess immunosuppressive activity, thus affecting the process of maturation and differentiation of DCs.[64] IL-6 and **m**acrophage **c**olony **s**timulating **f**actor (M-GSF) secretion by tumors inhibits differentiation of monocytes into mature DCs; IL-10 prevents the up-regulation of antigen-presenting and co-stimulatory molecules and production

of IL-12 by DCs and therefore, blocks the maturation of DCs.[65,66] DC maturation status is, therefore, a central point in anti-tumor immune response initiation. In their immature state, DCs exhibit a low expression of MHC and co-stimulatory molecules and consequently, are not able to stimulate the activation and differentiation of T cells.[49,67] Moreover, immature DCs can internalize apoptotic tumor cells and promote activation of Tregs, leading to an immunological tolerance and, consequently, to the failure of an effective anti-tumor response.[48,49]

Unlike the immature DCs, mature DCs play a crucial role in the initiation of the innate and adaptive anti-tumor immune responses. Hence, the efficiency and the ultimate success of anti-tumor immunity are dependent on proper maturation and activation of DCs.

1.2.3 DC-based vaccination procedures

1.2.3.1 DC precursor isolation

The development of cytokine-driven methods for expanding and differentiating DCs *ex vivo* has been explored in order to optimized DC-based immunotherapy. These methods include DC *in vitro* differentiation from monocytes, from $CD34^+$ hematopoietic stem cell progenitors or *in vivo* expansion of blood DCs.

The most common methodology used involves the *ex vivo* generation of DC from peripheral blood monocytes ($CD14^+$ cells), which are isolated from the patient. This method creates large amounts of immature monocyte-derived-DCs (mo-DCs) that are functionally similar to blood immature DCs. Moreover, in contrast to $CD34^+$ derived DCs, there is no need for **g**ranulocyte **c**olony-**s**timulating-**f**actor (G-CSF) or **F**MS-**l**ike **t**yrosine **k**inase **3** **l**igand (Flt3L) administration to the patient, in order to mobilized $CD34^+$ cells. First, **p**eripheral **b**lood **m**ononuclear **c**ells (PBMCs) are obtained from leukapheresis procedure and then monocytes are isolated, typically, either by **$CD14^+$** positive immune-magnetic **s**election (CD14-S) or by **p**lastic **a**dherence **s**election (PA-S). Monocytes are then cultured for 5-7 days in the presence of IL-4 and **g**ranulocyte **m**acrophage **c**olony-**s**timulating **f**actor (GM-CSF) to differentiate into immature DCs.[48,68-70]

Although both CD14-S and PA-S are two well-established and efficient enrichment-monocyte methods, the mo-DCs generated by these two methods show some phenotypic and

functional differences between each other.[71] CD14-S is a faster method and originates higher yields of pure $CD14^+$ cell population.[71] On the other hand, Elkord and co-workers[72] reported that mo-DCs generated by the PA-S method exhibit a higher IL-12, IL-10 and TNF-α secretion and, consequently, a higher ability to induce cytotoxic Tc1 and Th1 subsets, than CD14-S mo-DCs. While both CD14-S and PA-S methods have been employed in several DC-based vaccine clinical trials, administered mo-DCs generated by PA-S method show a poor capacity to transmigrate to the target tissues.[73,74]

1.2.3.2 Maturation of the *ex vivo* generated DCs

The *ex vivo* generated immature DCs are further stimulated to become mature, by culturing for an additional 1-2 days in the presence of maturation stimuli. The most common maturation stimulus used in DC-immunotherapy is a mixture of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and **P**rosta**g**landin **E2** (PGE2)), which induces increased expression of MHC-class I/II, CD40, CD80 and CD86 molecules, coherent with the DC mature phenotype, as well as the expression of CCR7, necessary for DCs migration to lymph tissues.[68,75] In addition to this approach, agonists of TLRs or CD40 ligands can also be used, although these approaches are not as effective to induce DC maturation as the pro-inflammatory cytokine cocktails.[48]

1.2.3.3 Tumor-antigen loading of the *ex vivo* generated DCs

In order to produce a long lasting and specific anti-tumor response, DCs have to be able to present tumor antigens to $CD4^+$ and $CD8^+$T cells. To date, several approaches to pulse DCs with tumor antigens have been explored. These include the use of patient's tumor-peptides/proteins or irradiated/lysed whole tumor cells. Another approach involves the use of bacterial or viral vectors or DC transfection with mRNA containing genes encoding tumor antigens. These tumor antigens after internalization and processing are presented via MHC-Class I/II, and ideally are able to induce the activation of a cytotoxic $CD8^+$ T cell response. During the immunotherapy procedure, the DC tumor-antigen-loading step can occur before or after the DC maturation step.[48,69]

Finally, the mature and tumor-antigen pulsed-DCs are re-administered to the patient, migrating subsequently to the lymph nodes, where they stimulate the patient's T cells and thereby allow eradication of the tumor cells (**Figure 8**).[48,59]

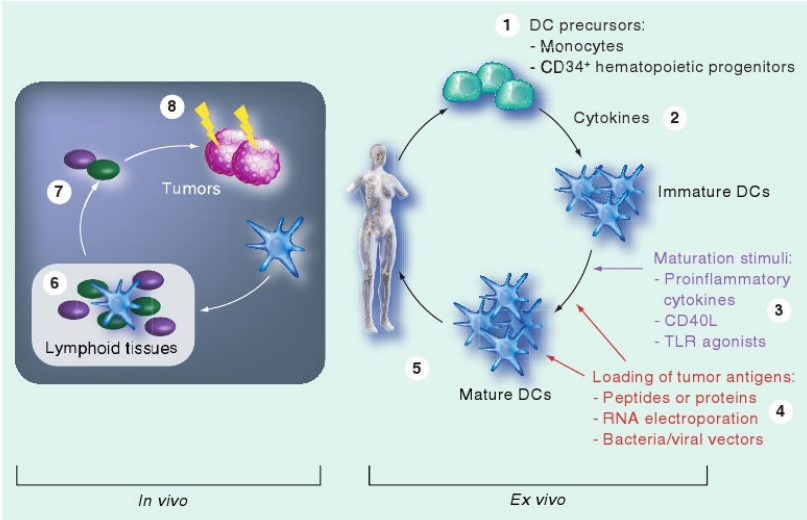

Figura 8- Dendritic cell immunotherapy: (1) DC precursors are isolated from the patient. (2) Incubation of DC precursors with cytokines induces their differentiation into immature DCs. (3) A maturation stimuli is applied to induce DCs to become mature. (4) Loading of DCs with tumor antigens from the patient is performed either before or after the maturation process. (5) The antigen-pulsed and mature DCs are injected back into the patient, (6) migrating subsequently to the lymphoid tissues, where they activate T cells. (7) Activated T cells egress the lymphoid tissue and transmigrate to the tumor site, (8) where they will stop tumor growth. *In Directing dendritic cell immunotherapy towards successful cancer treatment. Sabado RL and Bhardwaj N. Immunotherapy. 2010.*

To date, several clinical trials involving DC-based vaccines have been conducted in patients with different types cancer.[48] Although these vaccines have been proven to be feasible, safe and to generate tumor-specific immune responses, the success of this immunotherapy has been limited.[76] Indeed, only one anti-cancer DC-based vaccine has been approved by **F**ood and **D**rug **A**dministration (FDA), the Provenge (Sipuleucel-T) from Dendreon pharmaceutical company, specific for advanced prostate cancer treatment.[77] The success of DC-based vaccination depends on a large number of variables that need to be optimized, namely the source of DC precursors, appropriate maturation stimuli, strategy for tumor-antigen loading and application route.[48] Importantly, the elimination of the tumor mainly depends on the type of cellular immunity elicited by DCs, i.e. generation of Th1/Tc1 vs Th2/Tc2 response. It is crucial that the *ex vivo* expanded DCs used in this type of therapy are fully mature and able to induce a Th1 response, needed for the activation of cytotoxic $CD8^+$ T cell activity, and therefore, vital for tumor clearance.[69] Equally important is the ability of these DCs to reach the target tissues from the vasculature, after administration into the patient. Thus, an immediate prerequisite to accomplish the enormous promise of DC-based vaccines in cancer treatment is to develop methodologies to optimize DC maturation status and their ability to elicit Th1 responses, as well as to understand the effectors mediating physiologic trafficking of vascularly administered DCs to the target tissues.

1.3 Glycobiology and its effect on the immune response

All cells in nature present a vast repertoire of glycan structures at their cell surface. Indeed, glycosylation of cell surface or of intracellular structures is involved in several physiological and pathological processes, including the modulation of innate and adaptive immune responses.[78] Glycans are involved in processes such as the recognition of self-molecules, host-pathogen interactions, leukocyte trafficking, humoral immunity and activation of immune cells.[78,79]

1.3.1 Glycosylation

Glycan structures that are found in eukaryotic cells are defined based on the nature of the glycan binding to the target scaffold (lipid or protein), leading to the formation of many types of glycoconjugates. Thus, glycoconjugates can be classified into four main groups: proteoglycans, glycosphingolipids (glycolipids), glycosylphosphatidylinositol (GPI)-linked proteins and glycoproteins. [80] Additionally, glycoproteins can be further divided into *N*- or *O*-glycosylated proteins, based on the amino acid residue that binds to the glycan determinant. Therefore, an *O*-glycoprotein consists of a glycan covalently attached to the oxygen atom of a serine (Ser) or threonine (Thr) residue, within an amino acid sequence; whereas in *N*-glycosylation, the glycan is covalently bonded to the nitrogen atom of an asparagine (Asn) residue within the amino acid sequence Asn-X-Ser / Thr (where X represents any amino acid except proline (Pro)).[80,81]

1.3.1.1 *O*-Glycans Biosynthesis

The *O*-glycosylation process is characterized by a stepwise sugar addition that occurs in the Golgi apparatus and involves a broad array of enzymes (**Figure 9**). There are different classes of *O*-glycans, but the most ubiquitous type of initial *O*-glycosylation involves the addition of an *N*-acetylgalactosamine (*O*-GalNAc) to a Ser or Thr residues, known as mucin type *O*-glycans.[82] This synthesis is initiated by one of the *N*-acetylgalactosaminyltransferase family members (ppGalNAcTs), forming the Tn antigen. The Tn antigen can be sialylated by the action of the α(2,6)sialyltransferases ST6GalNAc-I/II, yielding sialyl-Tn antigen (sTn) and inhibiting further sugar addition. More frequently, the GalNAc residue of Tn can be elongated by core 1 β(1,3)galactosyltransferase (Core1GalT or T synthase) or by core 3 β(1,3)*N*- acetylglucosaminyltransferase (C3GnT), creating the core 1 structure, also known as

T antigen (Galβ1-3GalNAc-Ser/Thr), or core 3 structure (GlcNAcβ1-3GalNAc-Ser/Thr), respectively. Core 1 can be further lengthened by the addition of an *N*-acetylglucosamine (GlcNAc) through core 2 β(1,6)*N*-acetylglucosaminyltransferase (C2GnT-1), forming core 2 structure. On the other hand, T antigen (core 1 structure) sialylation, by the α(2,3)sialyltransferases ST3GalI/IV or α(2,6)ST6GalNAc II (forming sialyl-T (sT) or sialyl-6T (s6T) antigens, respectively), stops core 2 formation. In contrast, repetitive additions of galactose (Gal) and GlcNAc by β(1,3/4)GalT and β(1,3/4)GnT enzymes, respectively, can further elongate core 2 structures, leading to type 1 (Gal linked to GlcNAc through a β(1,3) linkage) and type 2 (Gal linked to GlcNAc through a β(1,4) linkage) lactosamine chain formation. [81-85]

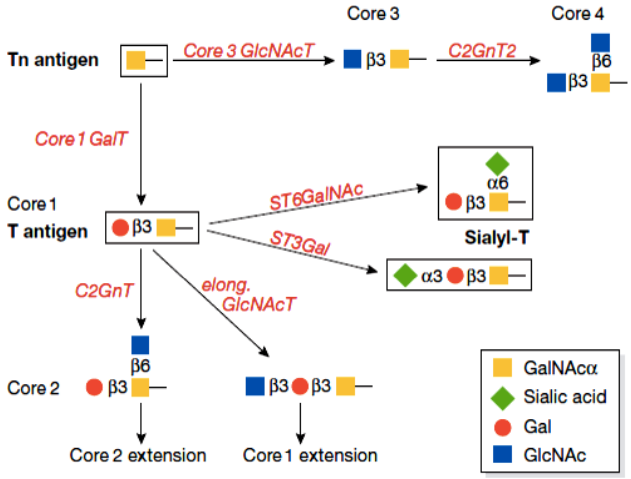

Figure 9- Schematic representation of *O*-linked glycoprotein synthesis pathway. *O*-glycan synthesis is initiated by an *N*-acetylgalactosaminyltransferase that transfers an *N*-acetylgalactosamine residue (GalNac) to a serine or threonine, forming the Tn antigen. Tn antigen can be sialylated by the activity of the α(2,6)-sialyltransferase ST6GalNAc-II, yielding the sialyl-Tn antigen (sTn), which ceases further sugar modification. Alternatively, the transfer of galactose (Gal) to Tn antigen leads to the formation of T antigen (core 1) by Core 1GalT. T antigen sialylation can be catalyzed by α(2,3)-sialyltransferase ST3GalI/IV or α(2,6)-sialyltransferase ST6GalNac-II, forming sialyl-T (sT) or sialyl-6T (s6T) antigens, respectively. In absence of sialylation, core 1 can be modified by the addition of an *N*-acetylglucosamine (GlcNAc) by C2GnT1, forming the "core-2" structure. Repetitive sequential and alternating additions of Gal and GlcNAc, respectively, create the lactosaminyl glycan chain that serves as an acceptor for terminal sialofucosylations. *In Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. Brockhausen I .EMBO reports. 2006.*

1.3.1.2 *N*-Glycans Biosynthesis

In contrast to *O*-glycosylation, the *N*-glycosylation process requires the production of an oligosaccharide precursor ($GlcNAc_2Man_5$) in the cytoplasmic phase of the endoplasmic reticulum (ER) membrane. This glycan is transferred en block from a lipid donor (Dolichol-phosphate (Dol-P)) to the Asn residue of a newly synthesized protein in the lumen of the ER and further processed in the Golgi compartment (**Figure 10**).[86] Briefly, *N*-glycan synthesis is initiated on the cytoplasmatic side of ER by the transfer of a GlcNAc-1-P to Dol-P, generating Dol-P-P-GlcNAc that is further elongated, by the addtition of GlcNac and mannose (Man) residues, into Dol-P-P-$GlcNAc_2Man_5$. This glycan precursor is flipped across the ER membrane to luminal side, where Man and glucose (Glc) residues are added, forming the mature *N*-glycan precursor $Glc_3Man_9GlcNAc_2$-P-P-Dol. The mature *N*-glycan precursor is then transferred to an Asn residue (Asn-X-Ser/Thr sequon) on a newly synthetized protein by the multi-subunit protein complex, oligosaccharyltransferase (OST), as the protein is translocated into the ER. After this transfer, the *N*-glycan precursor goes through Glc and Man removal cycles by glucosidases and mannosidases in both ER and *cis* Golgi compartment until $Man_5GlcNAc_2$Asn structure is generated. For the biosynthesis of hybrid and complex *N*-glycans, further reactions are initiated in the medial-Golgi by the action of an *N*-acetylglucosaminyltransferase-1 (GlcNAcT-I/MgaT1), which adds an GlcNAc residue to the α(1,3)Man on the core of $Man_5GlcNAc_2$. The resulting structure may be elongated, giving rise to hybrid *N*-Glycans; or lose the two terminal Man units by mannosidase II, forming the $GlcNAcMan_3GlcNAc_2$ core. A second GlcNac residue is then added to the α(1,6)Man in the $GlcNAcMan_3GlcNAc_2$ core, by the action of GlcNacT-II, initiating the formation of complex *N*-Glycans.[87-90]

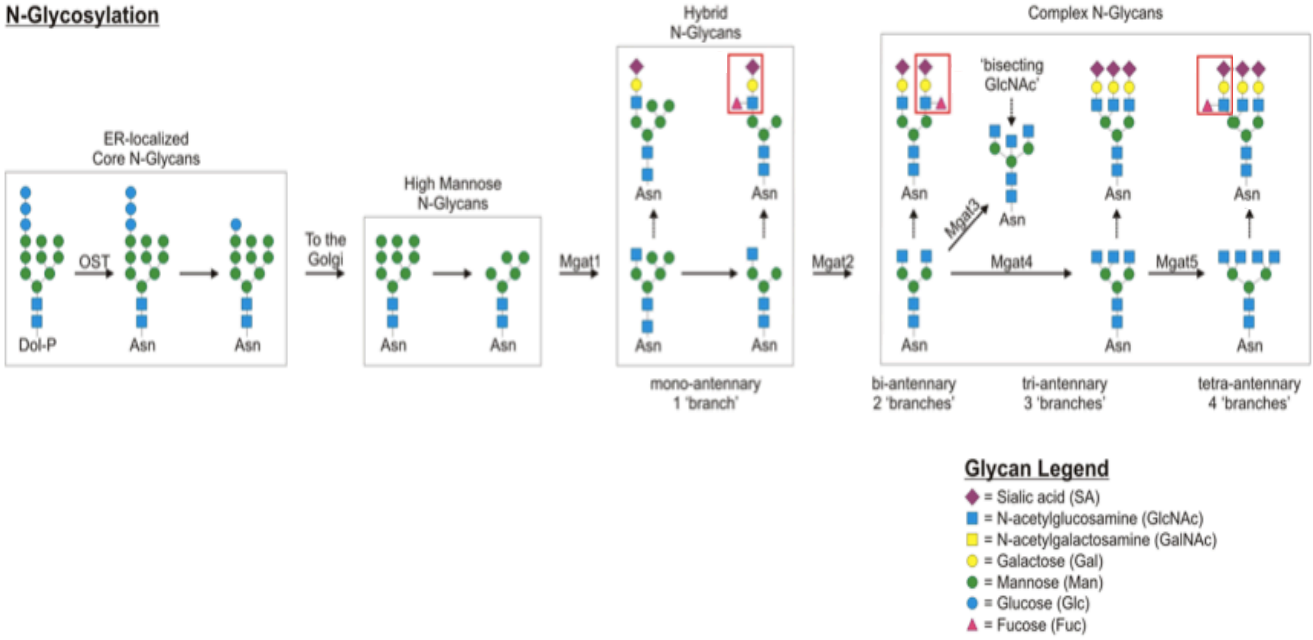

Figure 10- Schematic representation of *N*-linked glycoprotein synthesis pathway. The biosynthesis of *N*-glycoproteins is initiated within the endoplasmatic reticulum (ER) with the synthesis of a dolichol-linked precursor oligossaccharide that is transferred en block to a newly synthetized protein by the multi-subunit protein complex, oligosaccharyltransferase (OST). Subsequent *N*-glycan processing is carried out in the ER and then within Golgi apparatus. Biosynthesis of hybrid and complex glycans is initiated by the action of MgaT1, which adds a GlcNAc residue to the mannose (Man) present on the α(1,3)-arm of the $Man_5GlcNAc_2$ structure. The resulting structure can either lose two mannose units off the α(1,6)-arm of the core mannose, leading to the formation of complex *N*-glycans, or not lose these mannoses, generating hybrid *N*-glycans. *In The regulatory power of glycans and their binding partners in immunity. Johnson JL et al.,Trends Immunol. 2013.*

As mentioned previously, glycosylation modulates several immune events. Specifically, in this PhD thesis we investigated the glycoconjugate structures that mediate leukocyte extravasation to sites of inflammation and the modulation of DC functionality by sialylated glycans.

1.3.2 Leukocyte recruitment to sites of inflammation

To exert their effector immune functions, circulating leukocytes have to migrate from the vasculature to injured sites. Therefore, the mechanisms involving leukocyte migration are a crucial component of both innate and adaptive immune responses.[91]

1.3.2.1 Leukocyte transmigration: The Multi-step Model

Leukocyte recruitment involves a sequential and coordinated series of molecular actions mediated by adhesive interactions between circulating leukocytes and endothelial cells in post-capillary venules (**Figure 11**). To initiate the extravasation process, circulating leukocytes have to establish low-affinity and reversible interactions (tethering) with endothelial cells to roll on venular endothelium (step 1). These interactions allow leukocyte velocity to decrease in areas of high fluid shear stress. Leukocyte rolling allows the cells to become exposed to chemokines immobilized by glycosaminoglycans on the endothelial surface, leading to engagement of chemokine **G-p**rotein-**c**oupled **r**eceptors (GPCRs) on leukocyte cell surface (step 2), thereby triggering G-protein-coupled integrin activation. This leads to subsequent firm adhesion of leukocytes to the endothelium (step 3), mediated by the binding of leukocyte integrins, as the **v**ery **l**ate **a**ctivation **p**rotein **4** (VLA-4) and **l**ymphocyte **f**unction-associated **a**ntigen **1** (LFA-1), to their respective endothelial adhesion receptors, the **v**ascular **c**ell **a**dhesion **m**olecule **1** (VCAM-1) and **i**nter**c**ellular **a**dhesion **m**olecule **1** (ICAM-1).[92,93] Tight binding by activated integrins allows the leukocytes to extravasate across the endothelium (step 4), which can occur via migration between adjacent endothelial cells, involving the transient dismantling of endothelial junctions (paracellular migration), or via migration through individual endothelial cells (transcellular migration).[94,95]

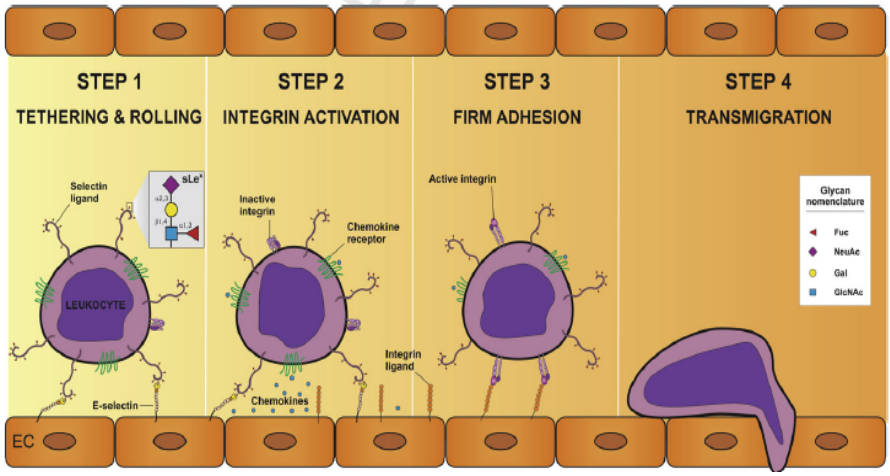

Figure 11– Multi-step model of leukocyte adhesion and migration along the vascular endothelium. Leukocytes engage in adhesive contacts with endothelial cells, through the binding of their sialofucosylated determinants to E-selectin expressed by endothelial cells (Step 1- Tethering and rolling). Engagement of chemokine receptors leads to integrin activation (Step 2) and subsequent firm adhesion of leukocytes to endothelium (Step 3), allowing their transmigration (Step 4). *In CD44 and HCELL: Preventing hematogenous metastasis at step 1. Jacobs PP, Sackstein R. FEBS letters. 2011.*

Although several cell-associated proteins are capable of mediating the first step of cell migration, the selectins are the most potent effectors of rolling and tethering interactions.[91,96]

1.3.2.2 Selectins and their carbohydrate ligands

1.3.2.2.1 The selectin family

The selectins are a family of three carbohydrate-binding proteins expressed on endothelial cells, leukocytes and platelets. Due to their requirement of calcium ions for binding, all three selectins, E-, P- and L-selectin, belong to the C-type lectins family (**Figure 12**).[97] Selectins share a common structure of five different domains: an *N*-terminal **c**arbohydrate **r**ecognition **d**omain (CRD), an **e**pidermal **g**rowth **f**actor like domain (EGF), a varying number of short **c**onsensus **r**epeats (CR) (3, 6, and 9 for L-, E-, and P-selectin, respectively), a transmembrane region and a C-terminal cytoplasmatic domain (**Figure 12**).[98] While the CRD and EGF domains are highly homologous between the three selectins, the structure of the transmembrane and cytoplasmic portions, as well as the extracellular CR domain are not conserved across the selectins, resulting in significant different molecular weights between selectins (L-selectin, 42 kDa; E-selectin, 64 kDa; P-selectin 86 kDa).[99,100]

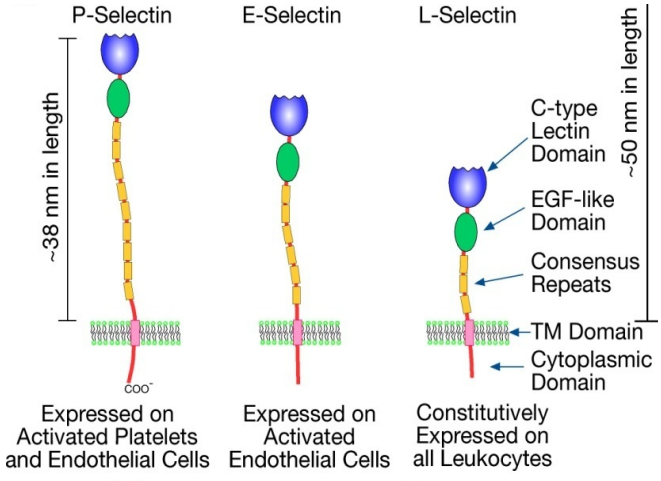

Figure 12- The selectin family. Selectins are a family of three carbohydrate binding proteins: P-selectin, expressed on activated platelets and endothelial cells, E-selectin expressed on activated endothelial cells and L-selectin expressed on leukocytes. The figure shows the five domains shared by selectins: C-type lectin domain, epidermal growth factor like domain (EGF), a varying number of short consensus repeats, a transmembrane region and a cytoplasmatic domain. *In Varki A et al. (Ed.), "Essentials of Glycobiology", Cold Spring Harbor Laboratory Press, 2nd Edition, 2009.*

Despite sharing common elements, the three selectins have different functions in diverse pathological and physiological processes and vary in their distribution and binding kinetics.

L-selectin (also known as **CD62** antigen-like family member **L** (CD62L), **l**eukocyte **a**dhesion **m**olecule **1** (LAM-1), **l**eukocyte-**e**ndothelial **c**ell **a**dhesion **m**olecule **1** (LECAM-1), DREG-56, Ly-22, Leu8, TQ1 and Mel-14 antigen) was first described by Gallatin and co-workers[101] in 1983 as a lymphocyte homing receptor. This molecule is found to be upregulated on hematopoietic stem cells and mature leukocytes, including all myeloid cells, subsets of natural killer cells, *naïve* T and B cells and central memory T cells.[97] However, its expression decreases on effector T cells after encountering antigen.[102] When leukocytes are activated, cell surface levels of L-selectin are regulated by proteolytic cleavage by metalloprotease-dependent shedding of the extracelulluar domain.[97]

P-selectin (also known as **CD62P**, **p**latelet **a**ctivation-**d**ependent **g**ranule to **e**xternal **m**embrane **p**rotein (PADGEM), **l**eukocyte-**c**ell **a**dhesion **m**olecule **3** (LCAM-3), **g**ranule **m**embrane **p**rotein 140 (GMP-140)) was discovered in 1984 by McEver and co-workers [103,104] and Furie and co-workers [105] as a glycoprotein expressed on the cell surface of activated platelets. P-selectin is constitutively expressed by megakaryocytes and endothelial cells, where it is stored in α-granules of circulating platelets and Weibel-Palade bodies, respectively. Following pro-inflammatory stimulus, by molecules such as thrombin or histamine, P-selectin is translocated from the granules to the cell surface by fusion of intracellular storage compartments with the plasma membrane. In murine endothelial cells, but not human, inflammatory mediators such as TNF-α, IL-1β and lipopolysaccharide (LPS) induce P-selectin mRNA transcription. [80,97,106] In these murine endothelial cells, TNF-α and IL-1β activation of P-selectin is mediated by the binding of the **n**uclear **f**actor **κ**-light chain-enhancer of activated **B** cells (NF-κB) and **a**ctivating **t**ranscription **f**actor-**2** (ATF-2) to the their response elements in the P-selectin promoter.[107] However, the promoter of P-selectin in primates lacks binding sites for NF-κB and ATF-2[108]. For this reason, in human endothelial cells, the only vascular selectin expressed post- TNF-α and IL-1β activation is E-selectin.[109]

E-selectin (also known CD62E, **e**ndothelial-**l**eukocyte **a**dhesion **m**olecule **1** (ELAM-1) or LECAM2) was discovered by Bevilacqua and co-workers[110,111] in the 1980´s as a leukocyte adhesion molecule on activated endothelial cells. In the majority of tissues, endothelial cells do not constitutively express E-selectin, being the expression of this protein strongly regulated by inflammatory cytokines, such as TNF-α and IL-1β. In affected areas, these

cytokines induce transient transcription (maximal expression at about 4 hours) of E-selectin mRNA in both human and mouse endothelial cells.[112] Cytokine-dependent activation of E-selectin is mediated by NF-κB binding to positive regulatory domains in the E-selectin promoter.[113] In contrast, skin and bone marrow microvessels express E-selectin constitutively.[114] Functionally, E-selectin slows leukocyte rolling to much lower velocities than either L- or P-selectin, favoring subsequent leukocyte arrest.[115] This capacity, along with the inability of human endothelial cells to upregulate P-selectin in the presence of IL-1β and TNF-α, is why E-selectin is considered to be the most important selectin for cell trafficking to sites of inflammation, as it plays a critical role in the recruitment of immune effectors to target inflammatory sites.

1.3.2.2.2 The carbohydrate E-selectin ligands

E-selectin recognizes a broad range of structurally diverse glycan epitopes that typically contain an α(1,3/4)-fucose (Fuc) and/or α(2,3)-sialic acid (Sia) modification(s), as shown in **Figure 13**.[114] The isomeric tetrassacharides sialyl Lewis^x (sLe^x - Siaα2-3Galβ1-4GlcNAcα1-3Fuc) and sialyl Lewis^a (sLe^a - Siaα2-3Galβ1-3GlcNAcα1-4Fuc) are the most common terminal epitopes present in E-selectin-binding determinants, however, structures related to sLe^x/sLe^a can also exhibit E-selectin binding activity.[116,117] Some sulfated derivates of Le^x and Le^a (3'-Sulfo-Lewis^x (3'-Sulfo-Le^X) and 3'-Sulfo-Lewis^a (3'-Sulfo-Le^a), respectively) were shown to bind to E-selectin, as well as an internally fucosylated sLe^x isomer, VIM-2. Core-2 based lactosamine structures, such as GalNAc-Lewis^x, or polylactosamine chains, in which fucose modifications occur at more than one GlcNAc residues (Tri-fucosyl-sialyl Lewis^x and Di-fucosyl-sialyl Lewis^x) can also support E-selectin-dependent adhesion.[97,98,118,119] However, it is important to point out that sLe^a and the sulfated derivates of Le^x and Le^a are not natively expressed in leukocytes, and therefore are not involved in leukocyte trafficking; although they have been shown to present E-selectin binding activity in the laboratory.[118,119] The sulfated derivates of Le^x and Le^a and sLe^a antigens are mostly expressed by cancer cells and are correlated with a high metastatic potential.[120-122]

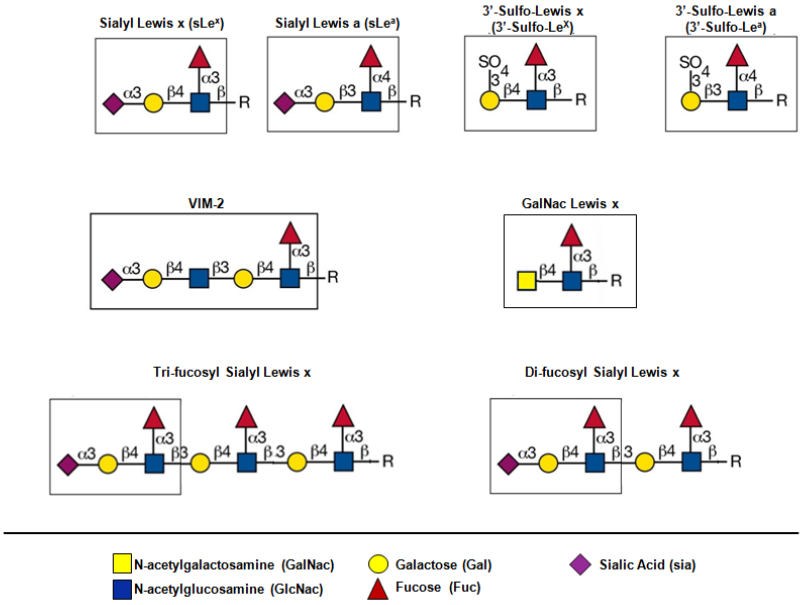

Figure 13- E-selectin reactive glycan structures. Sialofucosylated and fucosylated glycan structures that terminate *O*-linked, *N*-linked or lipid-linked glycoconjugate structures, and that have been shown to display E-selectin biding activity. *Adapted from Varki A et al. (Ed.), "Essentials of Glycobiology", Cold Spring Harbor Laboratory Press, 2nd Edition, 2009.*

1.3.2.2.3. Glycosyltransferases associated with biosynthesis of selectin carbohydrate-binding determinants

E-selectin binding epitopes are regulated by enzymes that directly alter sialylation and fucosylation of terminal lactosamines. These determinants are typically displayed at the end of *O*-glycans, *N*-glycans or glycolipid precursor structures. The synthesis of sialofucosyltated epitopes requires the coordinated and sequential action of specific glycosyltransferases localized in the lumen of the Golgi apparatus. Complete formation of E-selectin binding determinants is catalyzed by the addition of Fuc through α(1,3/4)fucosyltransferases and of Sia by α(2,3)sialyltransferases on type 1 (Gal linked to GlcNAc through a β(1,3) linkage – (s)Le^a structures) or type 2 (Gal linked to GlcNAc through a β(1,4) linkage – (s)Le^x structures) lactosamine chains (**Figure 14**).[123,124] So far, six human fucosyltransferases (FT) have been found to catalyze the addition of Fuc in an α(1,3/4) linkage to GlcNAc - FTIII, FTIV, FTV, FTVI, FTVII and FTIX. Each enzyme exhibits specificity for acceptor substrates and, therefore, have the ability to generate distinct fucosylated structures.[125] Specifically, FTIII and FTV are unique in that they exhibit both α(1,3/4) fucosyltransferase activity on both sialylated and unsialylated lactosamines. Both enzymes can synthesize sLe^x and sLe^a structures, as well as the unsialylated forms, Le^x and Le^a. FTVI has been reported to synthesize Le^x and sLe^x, whereas FTVII can only synthesize sLe^x. The α(1,3)

fucosyltransferases FTIV and FTIX are known to only synthesize Le^x.[125] The sialylated forms of Lewis antigens are synthesized by the action of the α(2,3) sialyltransferases. These enzymes transfer sialic acid residues to the Gal on the lactosamine chain, exclusively acting prior to fucosylation of the lactosamine chains.[83,126] There are 6 members of α(2,3) sialyltransferase family- ST3Gal I to ST3Gal VI – but only ST3GalIII, ST3GalIV, and ST3GalVI are reported to contribute to the creation of $sLe^{x/a}$.[127] Importantly, ST3GalVI exclusively catalyzes the formation of sLe^x, while ST3GalIII and ST3GalIV can additionally synthesize sLe^a. [128-130] The sialyltransferase ST3GalIII exhibits preference for type 1 lactosamine chain receptors whereas ST3GalIV preferentially acts on type 2 oligosaccharides.

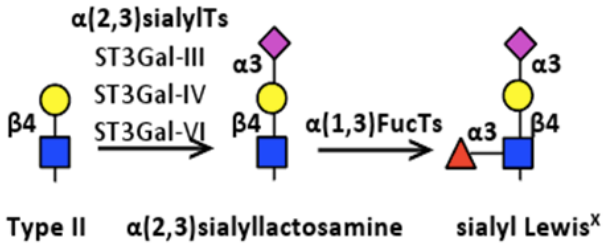

Figure 14- Sialyl $Lewis^X$ synthesis. The α(2,3)-sialyltransferases, ST3GalIII, IV and VI, terminate the elongation of both *O*- and *N*-Glycans by creating sialylated lactosamine chains that can be further fucosylated by the action of the fucosyltransferases FTIII, V, VI and VII, creating sLe^x.

Of the six α(1,3/4)fucosyltransferases, only three are thought to be expressed in human leukocytes, FTIV, FTVII and FTIX.[125,131] Studies have shown that leukocytes from mice lacking FTVII have deficient adhesive interactions with E- and P-selectin, suggesting this fucosyltransferase plays a predominate role in E-selectin ligand biosynthesis.[132,133] However, similar studies using mice deficient in both FTVII and FTIV have indicated that FTIV also contributes to leukocyte E- and P-selectin ligand expression, being crucial for slow leukocyte rolling velocity.[134,135] Importantly, in contrast to FTVII, E-selectin binding activity conferred by FTIV fucosylation occurs mainly on glycolipids, rather than glycoproteins.[136] Interestingly, a recent study suggested that, unlike murine leukocytes, FTIX plays a significant a role in E-selectin dependent interactions in human myeloid cells, where FTVII and FTIV contribute to a lesser extent.[131]

For the α(2,3) sialyltransferases, studies indicated that leukocytes from mice deficient in ST3GalIII preserved E- and P-selectin binding activity.[137] However, leukocytes from ST3GalIV null-mice show a partial loss of the ability to bind to both E- and P-selectin *in vitro*, suggesting that another α(2,3)sialyltransferase, most likely ST3GalVI, collaborates

with ST3GalIV in the selectin ligand biosynthesis process.[137] In agreement with murine studies, ST3GalIV also plays an important role in the biosynthesis of E- and P-selectin ligands in human leukocytes.[138]

Glycosyltransferases involved in biosynthesis of *O*-glycans have also been studied for their contributions in the generation of functional selectin ligands using knockout mouse models. One study reported that neutrophils from mice deficient in ppGalNAcT-1 showed impaired recruitment during inflammation, due to a significant reduction in E- and P-selectin ligand levels.[139] Mice deficient in C2GnT-1 showed reduced E-selectin and P-selectin binding activity of neutrophils under static and shear-based rolling assays.[140-142] β(1,4)GalT-I-deficient mice showed reduced inflammatory responses and impaired neutrophil P-selectin binding activity, however the contribution of this enzyme in the synthesis of E-selectin counter-receptors remains to be elucidated.[143]

1.3.2.3 Glycoconjugates structures for E-selectin ligands

Several glycostructures with E-selectin binding activity have been identified on various hematopoietic cells. Here, we review the glycoconjugates that act as scaffolds for functional E-selectin ligands on human and mouse leukocytes.

Cutaneous lymphocyte antigen (CLA)

CLA is the E-selectin reactive glycoform of **P**-selectin **g**lycoprotein **l**igand-**1** **(**PSGL-1). PSGL-1 is a transmembrane 240-kDa homodimeric, mucin-like glycoprotein expressed on leukocytes and some activated endothelial cells that plays a crucial role in the homing of leukocytes into inflamed tissue.[144,145] E-selectin binding activity of PSGL-1 is conferred by sialylated and fucosylated core 2 based *O*-glycans that are distributed along the extracellular domain.[146] Several studies have identified PSGL-1 as one of several scaffolds displaying glycan epitopes with E-selectin ligand activity on different human and mouse blood cell subsets, including human and mouse $CD34^+$ cells, human monocytes and DCs, and human and mouse neutrophilis and T cells.[145,147-151] PSGL-1 is also the dominant ligand for P- and L-selectin and is the only known glycoprotein that binds all three selectins.[152,153]

Hematopoietic cell E- and L- selectin ligand (HCELL)

HCELL is the sialofucosylated glycoform of CD44 that exhibits E-selectin binding activity. CD44, itself, is an 80-200 kDa transmembrane glycoprotein expressed by the majority of mammalian cells. It serves as receptor for **h**yaloronic **a**cid (HA) and participates in a broad range of cellular activities, including lymphocyte activation, leukocyte trafficking, hematopoiesis, cell growth and survival, and tumor dissemination.[154] The CD44 protein family displays high structural heterogeneity as a result of both alternative splicing of CD44 gene transcripts and post-translational modifications of the protein. The standard isoform of CD44 (CD44s or CD44H) is encoded by mRNA transcripts comprising exons 1–5 and 16–20 ("s1–s5 and s6–s10") and is ubiquitously expressed in mammalians cells. On the other hand, CD44 variant isoforms (CD44v) contain peptide products of variant exons (exons "v2–v10") in addition to standard exon peptide products. Post-translation modifications along with extensive alternative splicing allow the formation of multiple protein isoforms, expressed in a tissue-specific manner.[155,156] The standard CD44 molecule ranges in molecular weight from 80 to 110 kDa, whereas alternative splicing of variant exons produces CD44 isoforms ranging from 90 to ~200 kDa. Post-translation modifications include the addition of different glycan structures, namely glycosaminoglycans, *N*- and *O*-glycan structures.[157] The sialofucosylated glycoform of CD44 was first described on human hematopoietic stem and progenitor cells (HSPCs) by Dimitroff and colleagues[158], but not on mouse HSPCs.[149] Subsequently, HCELL was described to be highly expressed in hematologic[158,159] and solid malignancies,[160] as well as a potential E-selectin binding glycoprotein on human and murine neutrophils.[161] Importantly, for all human HSPCs and blast cells, sLe^x epitopes are exclusively displayed on *N*-glycan lactosamine structures on CD44s molecule; while, HCELL expressed by solid tumors displays E-selectin reactive carbohydrate epitopes on *O*-glycans of CD44 variant isoforms.[159,160] Because of its potent capacity to bind to selectins in high shear conditions, HCELL has been designated as the most potent L- and E-selectin ligand described on human cells. [158,162]

CD43E

CD43, also known as sialophorin or leukosialin, is a cell surface glycoprotein expressed by the majority of hematopoietic cells that is involved in several important processes, including cell development, activation, survival, and migration.[163-167] Glycosylation of CD43 molecules with either core 1 or core 2 *O*-glycan structures produces the 115 and 135 kDa glycoforms,

respectively.[168] E-selectin binding activity of CD43 was first reported on mouse Th1 cells by Matsumoto and colleagues [169] and on human T cells by Fuhlbrigge and colleagues.[170] Subsequently, murine neutrophils,[171] human and mouse $CD34^+$ cells[149] were also shown to express the E-selectin-reactive CD43 glycoform, CD43E. CD43 aberrantly decorated with $sLe^{x/a}$ determinants was also reported in colon cancer cells.[172] Importantly, the sLe^x epitopes that contain E-selectin binding activity of the CD43E glycoprotein are displayed on *O*-glycans.[149]

E-selectin ligand -1 (ESL-1)

ESL-1, also called MGF-160 or **c**ysteine-rich fibroblast growth **f**actor **r**eceptor **1** (CFR-1) is a 150 kDa transmembrane glycoprotein widely expressed and is well known for functioning as a fibroblast growth factor receptor, contributing to several cellular operations such as embryonic development, leukocyte migration, cell division and bone morphogenesis.[173,174] ESL-1 was identified as the chief E-selectin binding glycoprotein on murine myeloid cells[175] and on human metastatic prostate cancer cells.[176] Removal of *N*-glycans from this fibroblast growth factor receptor abolishes its E-selectin ligand activity.[177]

β2 integrins

β_2 integrins (CD11/CD18) are a family of leukocyte adhesion molecules that mediate cell-cell and cell-substrate interactions, playing a critical role during inflammatory events. β_2 integrin proteins are heterodimers composed of a β_2 chain (CD18 (~90 kDa)) and a variable α-subunit (CD11a (~177 kDa), CD11b (~165 kDa) or CD11c (~150 kDa)[178-182] The first evidence that CD11a/CD18 and CD11b/CD18 expressed by human neutrophils could act as ligand for the vascular E-selectin was reported by Kotovuori and colleagues,[183] and confirmed some years later by Crutchfield and colleagues.[184] Importantly, the E-selectin reactive sialofucosylated epitopes present in β2 integrins are displayed on *N*-glycans.[184] Zen and colleagues reported evidences that the CD11b and CD18 subunits are both decorated with sLe^x epitopes.[185]

MPO-EL

Myeloperoxidase (MPO) is a 150 kDa lysosomal peroxidase enzyme that is expressed in neutrophil and monocyte granules (azurophil granules). MPO is a dimeric protein consisting of two light chains (~12kda) and two heavy chains (~65 kDa) bound to a prosthetic heme group.[21,186] Recent studies have shown that MPO-EL, a glycoform of the heavy chain of MPO, is an E-selectin ligand on circulating G-CSF-mobilized leukocytes and on blood

myeloid cells in febrile leukocytosis patients. E-selectin binding activity of MPO-EL is conferred by sLe^x epitopes displayed on *N*-glycan structures on the heavy chain of MPO.[187]

Basigin (bsg)/CD147

Bsg is a ~40-70 kDa membrane immunoglobulin (Ig)-like glycoprotein ubiquitously expressed.[188] Recently, Noritoshi Kato and colleagues reported that murine neutrophils and HL-60 cells express a glycoform of bsg that contains E-selectin reactive sialofucosylated *N*-linked polylactosamine chains.[189]

L-selectin

As mentioned previously, L-selectin is a crucial player in lymphocyte homing to lymph nodes. This selectin can also function as an E-selectin ligand on human neutrophils [190] and cultured T lymphobasts[191], but not mouse neutrophils.[192] E-selectin binding activity of L-selectin is dependent on sLe^X bearing *O*-glycan structures. [192]

Glycolipids

Prior studies have suggested that sialofucosylated determinants displayed in lipid backbones can mediate adhesion to E-selectin. Tiemeyer and colleagues reported that leukocyte sialylated lactosylceramides decorated with internally fucosylated GlcNAc structures display ability to bind to E-selectin.[193] Studies focused on the digestion of surface proteins have supported this claim and indicate that extensive proteolytic cleavage of NK cells, eosinophils and neutrophils does not affect sLe^x expression and has no effect on E-selectin adhesion.[194,195] Moreover, sLe^x and sLe^a synthetic glycolipids were shown to mediate tethering and rolling of E-selectin–expressing cells in shear flow,[196] while abolishment of glycosphingolipid synthesis inhibited E-selectin binding to some extent.[197] More recently, sialylated lipid scaffolds extracted from breast cancer cells were described to also support binding of E-selectin-expressing cells under shear stress.[198]

1.3.3 DCs and glycosylation

DCs show a specific glycan profile at cell surface that can be modulated during development, maturation and immune regulation.[199] Typically, the terminal positions of eukaryotic cell surface glycoconjugates are occupied by the negatively charged and non-reducing

monosaccharide sialic acid.[200] Sialic acid content depends on the expression of a set of different sialyltransferases, localized at Golgi and/or sialidases (enzymes that cleave sialic acid linkages), located in the cytoplasm, membrane or lysosomes. These sialyltransferases transfer sialic acid from a donor substrate, *N*-acetylneuraminic cytidine monophosphate (CMP-Neu5Ac), to the terminal positions of the oligosaccharide chains and depending on their nature can catalyze different linkages, in particular α(2,3) or α(2,6) linkage to galactose residues and α(2,6) linked to GalNAc or GlcNAc residues.[127,201] These terminal sugars are involved in many cellular functions, both in physiological and pathological processes, including the regulation of the immune system and triggering progression of certain infections and diseases.[200,202] They can mask underlying structures, preventing cell-to-cell interactions through non-specific repulsive effects or preventing recognition by other molecules such as lectins.[126,203,204]

Besides the crucial role of sialic acids in migration of leukocytes, they play an important role in DC-pathogen interaction and recognition and are a key determinant in DC differentiation from monocyte precursors. Specifically, human mo-DCs display a high content of α(2,6) and α(2,3)sialic acid linked to carbohydrate moieties compared to their monocyte precursors. Furthermore, this increased sialylation is due to an upregulation of the sialyltransferases ST3Gal-I and ST6Gal-I.[205-207] Interestingly, DC maturation is also affected by sialic acid levels. Jenner and co-workers reported that mo-DCs with a higher α(2,6)sialic acid linked content exhibit a more tolerogenic and immature phenotype.[206] Consistently, removal of sialic acid from cell-surface of mo-DCs cells improves their maturation status and the inactivation of the sialyltransferases ST3Gal-I and ST6Gal-I induces a more mature phenotype in murine DCs.[208,209]

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Chapter 2

Rationale and specific objectives

2. Rationale and specific objectives

Glycosylation is the most common and multifaceted form of protein post-translational modification. It has been shown that protein glycosylation interferes with different immunological mechanisms and proper glycosylation may be a requisite for normal immune function. Therefore, understating of how glycosylation influences immunity is actually crucial for the complete comprehension of some immune related-functions and may provide key insights for the development of novel immunotherapeutic approaches in the future. Specifically, in the present work we have been focused mainly in two immune events that are strongly modulated by glycosylation: leukocyte extravasation to sites of inflammation that is modulated by E-selectin reactive glycoproteins; and dendritic cell (DC) ability to prime T cell mediated-immune responses modulated by sialylated glycans.

Specific objectives

2.1. To identify the nature and structure of the E-selectin reactive glycoproteins that are expressed on human peripheral mononuclear blood cells (PBMCs)

The vascular E-selectin and its carbohydrate-ligands are one of the key mediators for leukocyte trafficking during inflammation. Although several glycoproteins have been shown to bind to E-selectin, the majority of the reports have focused on results obtained uniquely from studies of mouse leukocytes or hematopoietic cell lines. Therefore, mouse E-selectin ligands have generally been presumed to be reflective of human E-selectin ligands, and usually not confirmed. However, it has been reported significant differences between mice and human leukocyte migration. As such, a complete and detailed identification of the physiological ligands for E-selectin expressed on human leukocyte is vital for the understanding of leukocyte extravasation process during inflammation. In **chapter 3**, we aimed to identify and characterize the nature of E-selectin ligands expressed by human primary peripheral blood mononuclear cells*:* monocytes, $CD4^+$ and $CD8^+$ T cells, and B cells. Specifically, we intended to gain mechanistic and molecular insights into the differential ability of these cells to traffic into inflamed tissues during acute or chronic inflammatory conditions. To this end, we performed functional assays to analyze the ability of these cells to bind to E-selectin under hemodynamic flow conditions, combined to several biochemical and transcriptomic studies.

2.2. To elucidate the E-selectin ligands expressed by human and mouse DCs obtained using different methodologies, or isolated from different cellular sources

DCs are professional antigen-presenting cells, which are specialized in the initiation and regulation of immune response. Due to their capacity to elicit robust antitumor responses, DC-based therapy represents a powerful and promising approach for treatment of cancer. Yet, the suboptimal capability of DCs to reach the target tissue from the vasculature has contributed to the limited success of this immunotherapy. The most common source of DCs used in immune-therapeutics has been the *ex vivo* generation of DC from monocyte precursors. Therefore, in **chapter 4**, we addressed the nature and the biological function of the E-selectin ligands involved in transendothelial migration of human blood monocyte-derived DCs (mo-DCs), using mo-DCs generated by two well-established enrichment-monocyte methods employed for clinical DCs applications: monocyte enrichment via CD14-selection (CD14-S) and those generated following plastic adherence-selection (PA-S). Moreover, we intended to augment the expression of E-selectin ligands on human mo-DCs, via enforced exofucosylation reaction, and assessed the impact of this glycoengineering on their capacity to bind to E-selectin and to transmigrate.

Although human monocytes are generally obtained from peripheral blood, there has been an increasing clinical interest in monocytes obtained from umbilical cord blood source. Therefore, in **chapter 5**, we aimed to perform a comparative study about the E-selectin ligand expression on adult peripheral blood (APB) and umbilical cord blood (UCB) monocyte-derived DCs. To identify the species-specific diversity among DC-E-selectin ligands, we also characterized the E-selectin reactive glycoproteins expressed by murine DCs. To achieve the appointed goals, we performed several functional assays (Transendothelial migration and parallel-plate flow chamber assays), coupled with biochemical studies, including western blot, immunoprecipitation, and flow cytometry methodologies. Collectively, in both **chapter 4 and 5**, we aimed to provide fundamental findings for the creation of strategies to improve the optimal deliver of DCs into target tissues.

2.3. To assess whether sialic acid removal from DC surface improves DC-mediated anti-tumor immune responses

Another obstacle to achieve the promising success of DC-based vaccines is establishment of efficient approaches that could successfully enhance maturation and cross-presentation ability of DCs to trigger specific cytotoxic T cell responses. Notably, it is now well known that high expression of cell surface sialic acid is tightly linked to an immature and tolerogenic phenotype of DCs. Therefore, in **chapter 6** we aimed to assess whether removal of sialic acid from human and mouse DC surface would result in improved ability of DCs to elicit tumor-specific immune responses. To this end, we assessed several features of DC immunobiology after sialic acid removal, including: DC maturation, direct and cross-presentation ability, secretion of Th1-inducing cytokines, activation of $CD4^+$ and $CD8^+$T cells and induction of specific tumor cell death.

Chapter 3

Comprehensive Analysis of Glycoprotein E-selectin Ligands Expressed on Human Peripheral Blood Mononuclear Cells

Paper I- ***submitted to blood***

TITLE

Comprehensive Analysis of Glycoprotein E-selectin Ligands Expressed on Human Peripheral Blood Mononuclear Cells

RUNNING TITLE

Expression of E-selectin ligands on human PBMCs

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KEY POINTS:

(1) There is wide diversity in the expression and structure of E-selectin ligands on native human peripheral blood mononuclear cells

(2) Human blood monocytes display multiple E-selectin ligands and exhibit potent E-selectin binding activity

Abstract

Immunity to incipient infection critically depends on the ability of sentinel circulating cells to infiltrate affected sites, a process which is initiated by leukocyte binding to E-selectin at inflamed endothelial beds. To evaluate the capacity of human peripheral blood mononuclear cells (PBMCs) to engage E-selectin, we performed E-selectin adherence assays under hemodynamic flow conditions coupled with flow cytometry assessment of E-selectin binding and western blot analysis of glycoprotein E-selectin ligands. Circulating monocytes uniformly displayed high expression of the canonical sialofucosylated E-selectin binding determinant sLe^X and markedly greater adhesive interactions with E-selectin than did lymphocytes, and although $CD4^+$T-cells exhibited significant E-selectin binding, $CD8^+$T and B-cells each showed minimal E-selectin engagement. Western blot analysis of monocytes revealed sLe^X determinants prominently on proteins PSGL-1, CD43, and CD44 (rendering the E-selectin ligands CLA, CD43E, and HCELL, respectively), whereas $CD4^+$T-cells expressed lower levels of CLA, CD43E, and HCELL, and $CD8^+$T-cells displayed no HCELL and very low amounts of CLA and CD43E. In contrast, circulating B-cells lacked E-selectin ligands, and quantitative PCR gene expression studies of enzymes that regulate display of sialofucosylated glycans showed correspondingly high transcript levels among monocytes and low levels among B-cells. Notably, α-(1,3)-exofucosylation markedly increased expression of functional E-selectin ligands among all PBMC subsets, most extensively on monocytes, with cell type-dependent specificity in the protein scaffolds that are targeted to create E-selectin ligands. Collectively, these findings unveil distinct patterns of E-selectin binding activity among PBMCs and provide key mechanistic insights into the molecular basis of differential E-selectin ligand expression among such cells.

Introduction

Under physiologic blood flow conditions, circulating immune cells extravasate at sites of inflammation via a multistep cascade of events. This process typically occurs at post-capillary venules, initiated by leukocyte tethering and rolling contacts on endothelium, followed by engagement of chemokine receptor(s) and G-protein-coupled integrin activation with subsequent firm adhesion, culminating in extravasation. The initial adhesive interactions are largely dictated by leukocyte binding to the vascular selectins, E- and P-selectin, causing leukocyte tethering and rolling on target tissue endothelial cells.[1] In humans, E-selectin is the only selectin inducible by the inflammatory cytokines TNF-α and IL-1β,[2] thus playing a key role in the recruitment of cells at inflammatory sites. As such, those circulating leukocytes specialized to display high avidity E-selectin ligands serve as primary sentinels of host defense.

E-selectin binds to sialofucosylated glycan determinants decorating specific glycoproteins and glycolipids. These glycans contain an α(2,3)-linked sialic acid substitution on galactose and an α(1,3)-linked fucose modification on *N*-acetylglucosamine, prototypically displayed as the terminal lactosaminyl tetrasacharide known as sialyl Lewis X (sLe^X).[1] Although both glycoproteins and glycolipids can display sLe^X, glycoproteins play a predominant role in initial binding to endothelial E-selectin under hemodynamic shear stress due to their extended membrane structure which yields more efficient primary contacts between flowing cells and the endothelium.[3]

To date, the vast majority of studies of E-selectin ligands have focused on neutrophils, particularly mouse neutrophils.[4] Among native human cells, the expression of E-selectin ligands has been most extensively characterized in hematopoietic stem and progenitor cells (HSPCs). Human HSPCs express three distinct glycoprotein E-selectin ligands: Cutaneous Lymphocyte Antigen (CLA), Hematopoietic Cell E-/L-selectin ligand (HCELL), and CD43-E, which are heavily sLe^X-decorated glycoforms of P-selectin Glycoprotein Ligand-1 (PSGL-1), CD44, and CD43, respectively.[5] Various studies of human neutrophils have shown a prominent role for CLA in E-selectin binding[6], however, our knowledge of the E-selectin ligands expressed by human peripheral blood mononuclear cells (PBMCs) is grossly incomplete. In particular, our lack of understanding regarding the function and structural biology of monocyte E-selectin ligands is surprising given that monocyte binding to E-

selectin is an essential prerequisite for inflammatory site recruitment and, subsequently, differentiation into pertinent macrophages or dendritic cells that shape the immune response.

In this study, we analyzed the E-selectin-dependent binding interactions of subsets of primary human peripheral blood mononuclear cells (PBMCs) with human endothelial cells under physiologic flow conditions. We found that human monocytes possess profound E-selectin binding activity and that $CD4^+$T-cells exhibit significant E-selectin binding, but $CD8^+$T-cells and B-cells have low/absent E-selectin binding activity. To elucidate the molecular basis of the observed marked differences in E-selectin binding activities, we performed complementary biochemical and functional studies of the E-selectin ligands expressed by these cells, as well as quantitative PCR gene expression studies of glycosyltransferases that mediate expression of sLe^X. Our studies reveal remarkable diversity in the structural biology of E-selectin ligands expressed on human leukocytes, provide key mechanistic insights on the function and formation of these structures, and broaden our understanding of the molecular effectors conferring immunosurveillance capabilities upon circulating human monocytes.

Materials and Methods

Isolation of blood cell subsets and cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from healthy donors under protocols approved by the Institutional Review Board of Brigham & Women's Hospital (BWH), with informed consent provided as per the Declaration of Helsinki. To isolate monocytes, PBMCs were obtained by Ficoll-Paque (Sigma-Aldrich) density gradient centrifugation followed by positive selection using anti-CD14 coated magnetic beads (Miltenyi).[7] $CD4^+$ and $CD8^+$T-cells and B-cells were isolated by negative selection using RosetteSep Human $CD4^+$, $CD8^+$, and B-cell enrichment cocktail, according to the manufacturer's instructions (StemCell Technologies). Cell suspensions were evaluated by flow cytometry for purity, which was routinely >90%.

Human umbilical vein endothelial cells (HUVECs) were obtained from the BWH Pathology Core facility and cultured as described.[8,9] Chinese hamster ovary (CHO) cells transfected

with full-length human E-selectin cDNA (CHO-E) and mock-transfected CHO cells (CHO-M) were cultured in modified Eagle's medium (MEM) supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin-streptomycin.

Antibodies and reagents used for flow cytometry

Cell surface expression of sLe^X, CD44, PSGL-1/CD162, and CD43 was assessed using mAbs HECA-452 (Biolegend), clone 2C5 (R&D Systems), KPL-1 and 1G10 (BD Biosciences), respectively. To evaluate post-selection cell purity, PBMC subsets were stained with FITC-conjugated-anti-human CD14 (Southern Biotech), APC-conjugated-anti-human CD4, FITC-conjugated-anti-human CD8 and cychrome-conjugated-anti-human CD19 mAbs (Pharmingen).

E-selectin reactivity was determined using recombinant mouse E-selectin-human Fc Ig chimera (E-Ig) (R&D Systems), in DPBS containing Ca^{2+}/Mg^{2+}, as previously described[5], with 5mM EDTA addition (calcium chelation) serving as staining control. Flow cytometry was performed using Cytomics FC 500 MPL flow cytometer (Beckman Coulter), with data analysis using FlowJo version 10.0.6 (TreeStar).

Preparation of whole cell lysates

Cells were suspended in 150mM NaCl, 50mM Tris-HCI (pH 7.4), 0.02% NaN_3, 20 µg/mL PMSF and protease inhibitor cocktail (Roche), sonicated, and then solubilized in 2% Nonidet P-40 (NP-40). For lysates undergoing immunoprecipitation with E-Ig, 2mM $CaCl_2$ was added.

Western blot analysis

Protein samples were boiled for 7 minutes in reducing Laemmli loading buffer (Boston BioProducts) and then resolved on 7.5% SDS-PAGE electrophoresis gels (Bio-Rad). Resolved proteins were transferred to PVDF membranes (Bio-Rad) and blocked with 10% milk and 0.1% Tween20 in TBS. Western blots were probed with primary antibodies (1µg/mL), followed by incubation with HRP-conjugated secondary antibodies (Southern

Biotech). For E-Ig blotting, membranes were incubated with E-Ig suspended in TBS 0.1% Tween20 containing 2mM $CaCl_2$ (1μg/mL), followed by staining with rat anti-mouse CD62E mAb (R&D Systems) and then goat anti-rat IgG-HRP (Southern Biotech). Antigens were detected by chemiluminescence using Lumi-Light Western blotting substrate (Roche).

Immunoprecipitation studies

Cell lysates were precleared with protein G-agarose (Invitrogen), followed by incubation with antibodies (anti-PSGL-1, anti-CD44, anti-CD43 (each from BD Biosciences)), anti-myeloperoxidase mAb (clone 03D03, Abcam), or E-Ig. Immunoprecipitates were then collected with protein G beads, beads were boiled, and released proteins were subjected to SDS-PAGE and western blotting.

Cell Surface biotinylation

Cells were incubated with Sulfo-NHS-SS-Biotin according to the manufacturer's instructions (Pierce Biotechnology). After cell lysis, biotinylated proteins were isolated with streptavidin beads (Invitrogen).

Blot rolling assay

The blot rolling assay was performed as described previously.[10] Briefly, western blots of CD44 immunoprecipitates were stained using HECA-452 mAb and rendered translucent by immersion in HBSS with 10% glycerol, 10 mM HEPES (pH 7.4) and 5 mM $CaCl_2$. Blots were then placed in a parallel-plate flow chamber, and CHO-E cells were perfused over blots at shear stress of 0.17 dynes/cm^2. Perfusion of CHO-M cells served as control to assess binding specificity.

Microfluidic adhesion assay

HUVEC monolayers were grown to confluence in microfluidic channels (Bioflux, Fluxion Biosciences). To stimulate expression of E-selectin, HUVECs were incubated with 40 ng/mL of TNF-α (R&D) for 4 hours at 37ºC. PBMC subsets were perfused through channels at an

initial shear rate of 0.5 dynes/cm^2, with step-wise increments to 8 dynes/cm^2. Rolling and arrested cells were counted. Binding specificity was assessed by treating PBMCs with sialidase or incubating HUVECs with function-blocking anti-E-selectin mAb.

Enzymatic treatments

To remove *N*-glycans, CD44 immunoprecipitates were treated with Peptide-*N*-Glycosidase (PNGase-F, New England Biolabs) according to the manufacturer's instructions. Sialic acid residues were removed by treatment with 200 mU/mL *Vibrio Cholerae* sialidase (Roche Molecular Biochemicals) at 37°C for 1 hour. Cell surface proteins were cleaved with 0.1% bromelain (Sigma-Aldrich) for 1 hour at 37°C, and efficiency assessed by flow cytometry staining for residual CD44 expression. For α-(1,3)-exofucosylation, cells were treated with 0.07 mg/mL of fucosyltransferase VII (FTVII) (R&D systems) in HBSS containing 10 mM HEPES, 0.1% human serum albumin and 1 mM GDP-fucose (Sigma-Aldrich), for 60 minutes at 37ºC. As controls, cells were suspended in buffer without FTVII (buffer-treated).

Isolation of RNA and Real-Time PCR

Total RNA from each sample was isolated using the RNeasy Micro Kit (Qiagen) and reverse-transcribed using the SuperScript VILO cDNA Synthesis kit and Master Mix (Applied Biosystems). Real-time PCR (RT-PCR) reaction was performed using SYBR Select Master Mix (Applied Biosystems). Primer sequences used for real-time PCR are listed in Supplemental Table 1 and gene expression was normalized to *GAPDH* (endogenous control) expression. The relative mRNA levels were calculated by adapting the $2^{-\Delta Ct} \times 100$ formula.[11]

Results

Native human monocytes display high E-selectin binding activity

To assess engagement of human PBMCs to vascular E-selectin under hemodynamic flow conditions, tethering/rolling adhesive interactions of monocytes, $CD4^+$ and $CD8^+$T-cells and B-cells were examined on TNF-α-stimulated HUVEC monolayers using a parallel-plate flow chamber (**Figure 1a**). Within the physiologic shear stress range of 1-4 dynes/cm^2, monocytes uniformly exhibited the highest tethering/rolling capacity, followed by $CD4^+$T-cells. Notably, monocyte adherence to HUVEC was maintained at steady levels at upwards of 7 dynes/cm^2. $CD8^+$T-cells showed minimal endothelial interactions that peaked at 1-2 dynes/cm^2, whereas B-cells did not exhibit rolling interactions (**Figure 1a**). Pretreatment of PBMC subsets with sialidase or pre-incubating HUVECs with function blocking antibody against E-selectin each abrogated tethering/rolling interations of all PBMCs, confirming the specificity of E-selectin receptor/ligand interactions.

To determine whether the observed variations in tethering/rolling capacity correlated with differential expression of E-selectin ligands, flow cytometry was performed using E-Ig chimera to measure E-selectin binding activity and mAb HECA-452 to measure expression levels of the canonical selectin-binding determinant sLe^X. All blood monocytes exhibited robust reactivity with E-Ig and HECA-452 mAb (**Figure 1b**). Among lymphocytes, $CD4^+$T-cells exhibited greater E-Ig and HECA-452 reactivity than $CD8^+$T-cells, whereas B-cells showed minimal reactivity (**Figure 1b**).

To assess the relative contribution of glycoproteins versus glycolipids as E-selectin ligands, cells were treated with bromelain. All PBMC subsets showed a substantial reduction of HECA-452 reactivity after bromelain treatment **(Figure 1c)**, indicating that E-selectin binding determinants are preferentially expressed on proteins, not lipids.

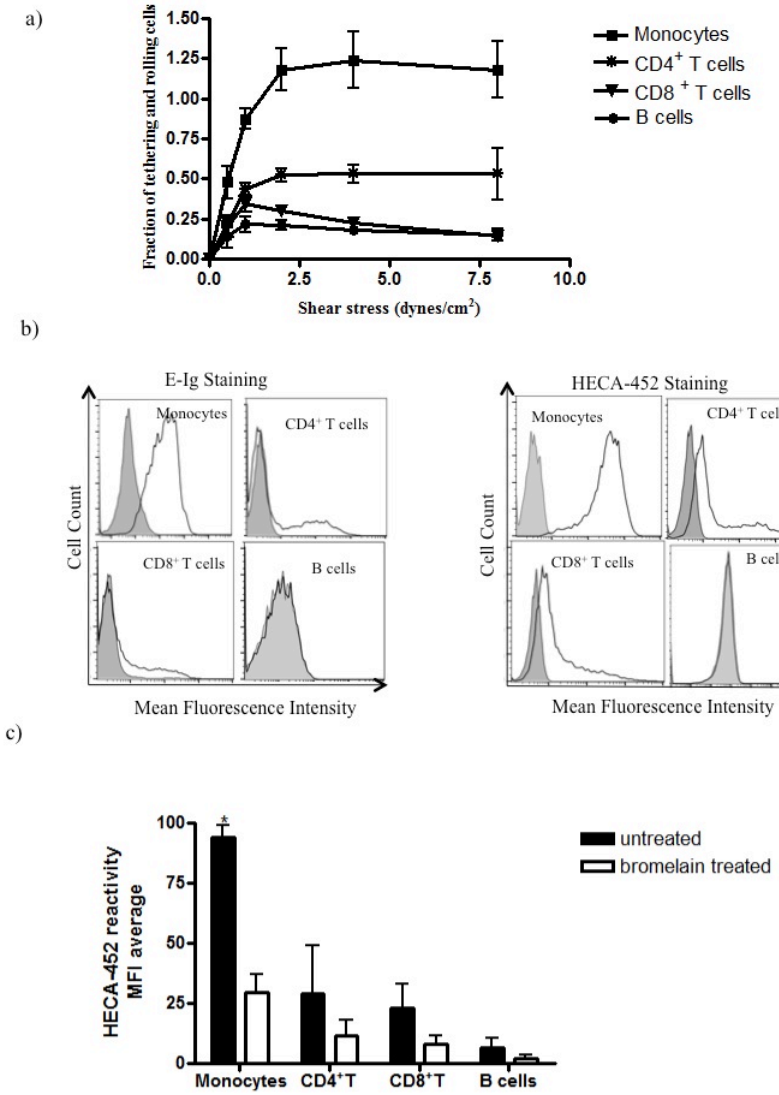

Figure 1. **Human monocytes exhibit greater E-selectin binding activity compared to lymphocytes. (a)** *Parallel-plate flow chamber analysis of tethering and rolling interactions of PBMCs on E-selectin-expressing endothelial cells*. Freshly isolated monocytes, $CD4^+$ and $CD8^+$T-cells, and B-cells were perfused over TNF-α-stimulated HUVECs in flow with shear stress ranging from 0.5 to 8 dynes/cm^2. Cell tethering and rolling was observed and video-recorded for analysis. The number of cells rolling on the HUVEC monolayer, as a fraction of total number of perfused cells, was quantified and averaged across three different fields of view at shear stress of 0.5, 1, 2, 4 and 8 dynes/cm^2. Monocytes and $CD4^+$T-cells engaged in rolling and adhesive interactions at a shear stress of up to 8 dynes/cm^2, whereas $CD8^+$ and B-cells only engaged in minimal shear-resistant adhesive interactions. The number of rolling cells for monocytes was three-fold greater than for $CD4^+$T-cells. Values are mean ± SEM; Data shown are from 5 independent experiments. **(b**) *Representative flow cytometry histograms of E-Ig (left) and HECA-452 (right) staining of monocytes, $CD4^+$ and $CD8^+$T-cells and B-cells*. Filled histograms represent incubation with isotype control or with E-selectin-Ig in the absence of Ca^{2+} (EDTA), and open histograms represent incubation with HECA-452 or E-Ig. As shown, human monocytes express the highest levels of E-selectin ligands, followed by $CD4^+$ and $CD8^+$T-cells, whereas B-cells lack E-Ig and HECA-452 reactivity. **(c)** *Bromelain treatment of PBMCs*. Untreated (black bars) or bromelain-treated (white bars) cells were stained with HECA-452 mAb and analyzed by flow cytometry. Bromelain digestion decreased HECA-452 reactivity in all PBMC subsets tested**.** Data shown are representative of 3 independent experiments.

Glycoforms of CD43, CD44, PSGL-1 and a 70 kDa protein are E-selectin ligands on human monocytes

To identify the molecular effectors of E-selectin binding by monocytes, E-Ig was used as probe in Western blot analysis. As shown in **Figure 2a**, under reduced SDS-PAGE conditions, three prominent E-Ig-reactive bands were observed at ~120-130 kDa, ~80-90 kDa and ~70 kDa. The monomer (i.e., reduced homodimer) of CLA migrates at ~120-130 kDa: CLA has been reported to be an E-selectin ligand on human monocytes[12,13] and flow cytometry confirmed surface expression of PSGL-1 on monocytes (**Figure 2b**). E-selectin ligands were then immunoprecipitated from monocyte lysates using E-selectin-Ig chimera, and analyzed by Western blot using anti-PSGL-1 antibody as a probe. As shown in **Figure 2c**, staining with anti-PSGL-1 mAb was observed at ~120-130 kDa, providing direct evidence of CLA expression.

Although not previously reported in monocytes, the sialofucosylated CD43 isoform - CD43E - has a molecular mass similar to CLA (~130 kDa) and functions as an E-selectin ligand in some human blood cells.[5,14] Flow cytometry confirmed the surface expression of CD43 on human monocytes (**Figure 2b**). The E-Ig immunoprecipitate also stained positive with anti-CD43 antibody, confirming that the ~120-130 kDa E-Ig-reactive band corresponds to both CD43E and CLA (**Figure 2c**).

HCELL, the E-selectin-binding glycovariant of CD44, migrates in reduced SDS-PAGE conditions as an ~80-90 kDa band.[15] We observed high CD44 expression on monocytes by flow cytometry (**Figure 2b**). Accordingly, E-Ig-immunoprecipitates of monocyte lysates were probed with anti-CD44 antibody on Western blots, and anti-CD44 mAb staining showed that the ~80-90 kDa band is HCELL (**Figure 2c**).

The E-Ig-reactive ~70 kDa glycoprotein did not stain with anti-PSGL-1, anti-CD43 or anti-CD44 mAbs and it is expressed on the cell surface of monocytes, as assessed by monocyte surface-biotinylation, followed by streptavidin pull-down of cell lysate material (**Supplementary Figure 1)**. A recent report suggested that G-CSF induces expression of an E-selectin-reactive glycoform of myeloperoxidase (designated "MPO-EL") with molecular mass ~65 kDa[16], however, MPO-immunoprecipitates from monocyte lysates did not stain with E-Ig, indicating that the E-Ig-reactive ~70 kDa protein is not MPO-EL (**Figure 2d**).

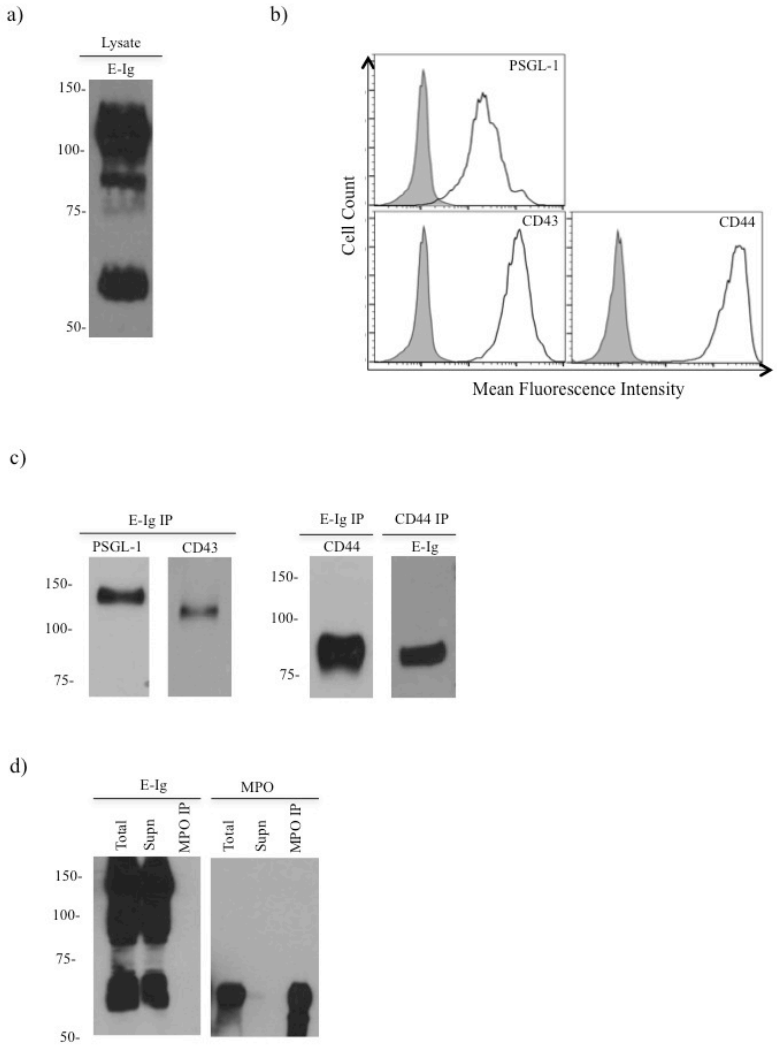

Figure 2. Identification of glycoprotein E-selectin ligands on human monocytes. (a) *Western blot analysis of whole cell lysate of human monocytes stained with E-Ig*. E-Ig staining of monocyte lysates resolved under reduced SDS-PAGE conditions revealed three principal bands at ~120-130 kDa, ~80-90 kDa, and ~70 kDa. **(b)** *Representative flow cytometry histograms of CD44, PSGL-1 and CD43 expression on human monocytes***.** Filled histograms represent incubation with isotype control and open histograms represent incubation with specific antibodies. As shown, human monocytes uniformly express CD44, PSGL-1 and CD43. **(c)** *Western blot analysis of glycoprotein E-selectin ligands of human monocytes*. E-Ig-immunoprecipitated (E-Ig IP) proteins from cell lysates of human monocytes were resolved by SDS-PAGE, blotted, and stained with anti-PSGL-1 and anti-CD43 antibodies (Left panel) or with anti-CD44 antibody (Right panel). In addition, CD44 was immunoprecipitated (CD44 IP) from cell lysates of human monocytes, resolved by SDS-PAGE, blotted, and stained with E-Ig (Right panel). Human monocytes express PSGL-1, CD43 and CD44 scaffolds that display E-selectin reactive epitopes. **(d)** *Western blot analysis of E-Ig-reactivity of human monocyte myeloperoxidase (MPO)*. MPO was immunoprecipitated from monocyte lysates, resolved by SDS-PAGE, blotted, and stained with anti-MPO mAb (right) or E-Ig (left). "Total" represents whole lysate and "Supn" corresponds to the cleared lysate, i.e., following MPO immunoprecipitation ("MPO IP"). As shown, MPO is not an E-selectin ligand on native human monocytes.

Human CD4$^+$T-cells natively express CLA, CD43E and HCELL, whereas CD8$^+$T-cells express only CLA and CD43E, and B-cells lack E-selectin ligands

To identify E-selectin ligands expressed on subsets of circulating lymphocytes, Western blot of CD4$^+$, CD8$^+$T-cell and B-cell lysates was performed using E-Ig as a probe. As shown in **Figure 3a**, E-Ig staining of CD4$^+$T-cell lysates revealed two distinct bands at ~120-130 kDa and ~80-90kDa, whereas staining of CD8$^+$T-cell lysates showed one band at ~120-130 kDa, and B-cell lysates lack E-Ig reactivity (**Figure 3a**).

Given the characteristic molecular weight of E-selectin-binding glycoforms of PSGL-1 and CD43 at ~120-130 kDa, and of CD44 at ~80-90 kDa, we performed flow cytometry to assess whether the relevant differences in E-Ig reactivity could reflect differential expression of these proteins among CD4$^+$T-cells, CD8$^+$T-cells, and B-cells. As shown in **Figure 3b**, all CD4$^+$ and CD8$^+$T-cells express, at similar levels, PSGL-1, CD43, and CD44. In contrast, B-cells lack expression of PSGL-1, only a small proportion (~5%) of cells express CD43, and CD44 is uniformly expressed but at levels lower than that of T cells (**Figure 3b)**.

As shown in **Figure 3c**, in CD4$^+$T-cells, PSGL-1 and CD43 immunoprecipitated from whole cell lysates showed robust staining with E-Ig, thus indicating expression of both CLA and CD43E. Immunoprecipitated CD44 was also slightly reactive with E-Ig, revealing a low level of HCELL expression in CD4$^+$T-cells. In lysates of CD8$^+$T-cells, PSGL-1- and CD43-immunoprecipitates each showed reactivity with E-Ig, however, in contrast to CD4$^+$T-cells, CD44-immunoprecipitates were not E-Ig reactive, indicating absence of HCELL expression (**Figure 3c**).

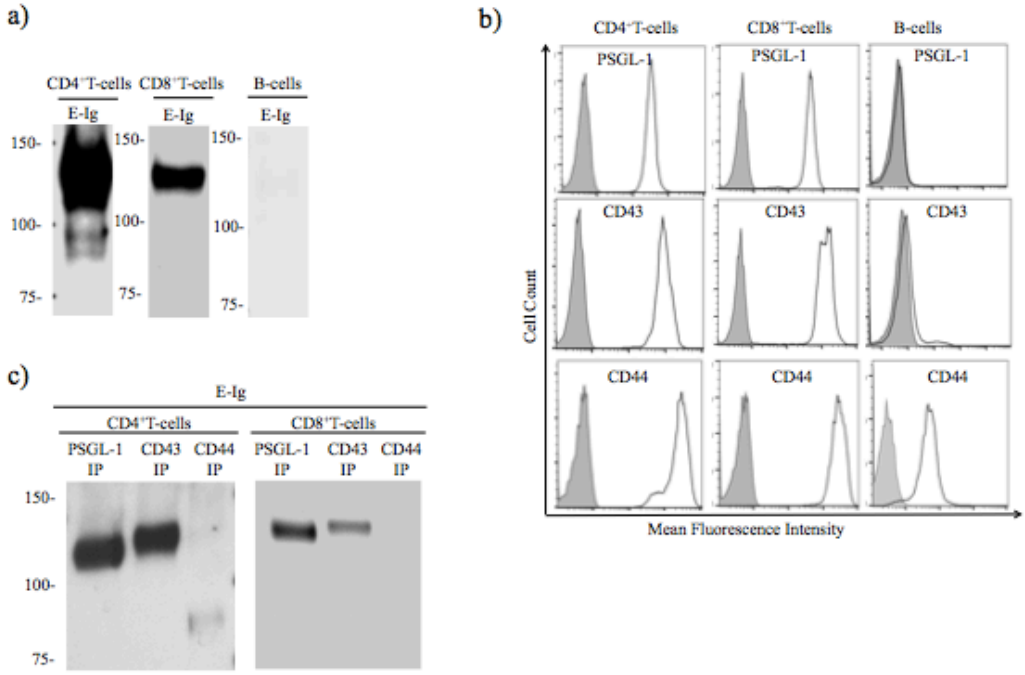

Figure 3. Identification of E-selectin ligands expressed on different subsets of lymphocytes. (a) *Western blot analysis of E-Ig-reactive proteins of human CD4*$^+$ *and CD8*$^+$*T-cells and B-cells*. Lysates were resolved by SDS-PAGE electrophoresis, and immunoblotted with E-Ig chimera. CD4$^+$ and CD8$^+$ T-cells show two and one E-Ig reactive band, respectively, whereas B-cells lack E-Ig staining. **(b)** *Flow cytometry analysis of PSGL-1, CD43 and CD44 expression on human lymphocytes*. Filled histograms represent incubation with isotype control and opened histograms represent incubation with specific antibodies. CD4$^+$ and CD8$^+$T-cells express high levels of PSGL-1, CD44 and CD43, whereas B-cells only express CD44 and a small amount of CD43. **(c)** *Identification of glycoprotein E-selectin ligands expressed on human T lymphocytes***.** PSGL-1, CD43 and CD44 were immunoprecipitated from whole cell lysates of human CD4$^+$ and CD8$^+$T-cells. Immunoprecipitates were then resolved by SDS-PAGE, blotted and stained with E-selectin-Ig chimera (E-Ig). Western blots reveal expression of E-selectin ligands CLA, CD43E and HCELL on human CD4$^+$T-cells, and CLA and CD43E on human CD8$^+$T-cells.

E-selectin binding determinants on HCELL are displayed on *O*-glycans on monocytes and *N*-glycans on CD4$^+$T-cells

HCELL expression has previously been reported only in human hematopoietic stem and progenitors cells (HSPCs)[15] and in certain types of tumors.[17] Structurally, the HCELL glycoform identified on human HSPCs consists of sialofucosylated determinants decorating *N*-glycans of standard CD44.[18] In contrast, tumors bearing HCELL glycoform display the relevant sialofucosylations on *O*-glycans.[17,19] To determine whether the E-selectin binding determinants of HCELL on human monocytes and CD4$^+$T-cells are displayed on *N*- or *O*-glycans, CD44 was immunoprecipitated and treated with PNGase-F, which cuts *N*-glycans

from the protein backbone. HCELL on KG1a cells presents its sLe^X determinants solely on *N*-glycans, and, as control for efficiency of *N*-glycan cleavage, CD44-immunoprecipitates from KG1a lysates were treated with PNGase-F; the characteristic loss of E-Ig reactivity was observed on western blot (**Figure 4a, left panel**). As expected from loss of sLe^X expression, sialidase digestion resulted in abrogation of E-Ig-reactivity in both monocytes and $CD4^+$T-cells **(Figure 4a)**. Notably, although PNGase-F treatment reduced the molecular weight of monocyte CD44, the intensity of E-Ig reactivity by western blot was not decreased, indicating that sLe^X determinants are not displayed on *N*-glycans of monocyte CD44 (**Figure 4a, middle panel**). However, in contrast, CD44-immunoprecipitates from $CD4^+$T-cells completely lost E-Ig reactivity following PNGase-F treatment, indicating that HCELL in these cells exclusively displays sLe^X on *N*-glycans (**Figure 4a, right panel**).

HCELL is a functional E-selectin ligand on both human monocytes and $CD4^+$T-cells

To further assess the capacity of HCELL of human monocytes and $CD4^+$T-cells to support E-selectin-dependent cell rolling under physiological shear conditions, blot-rolling assays were performed.[10,20] As shown in **Figure 4b**, CHO-E cells, but not CHO-M cells, exhibited rolling interactions on HCELL immunoprecipitated from monocytes and $CD4^+$T-cells. Taken together, these data confirm that HCELL is a functional E-selectin ligand in both monocytes and $CD4^+$T-cells. Importantly, although sLe^X epitopes are displayed on *O*-glycans of CD44 on monocytes, this does not affect monocytes' HCELL functionality.

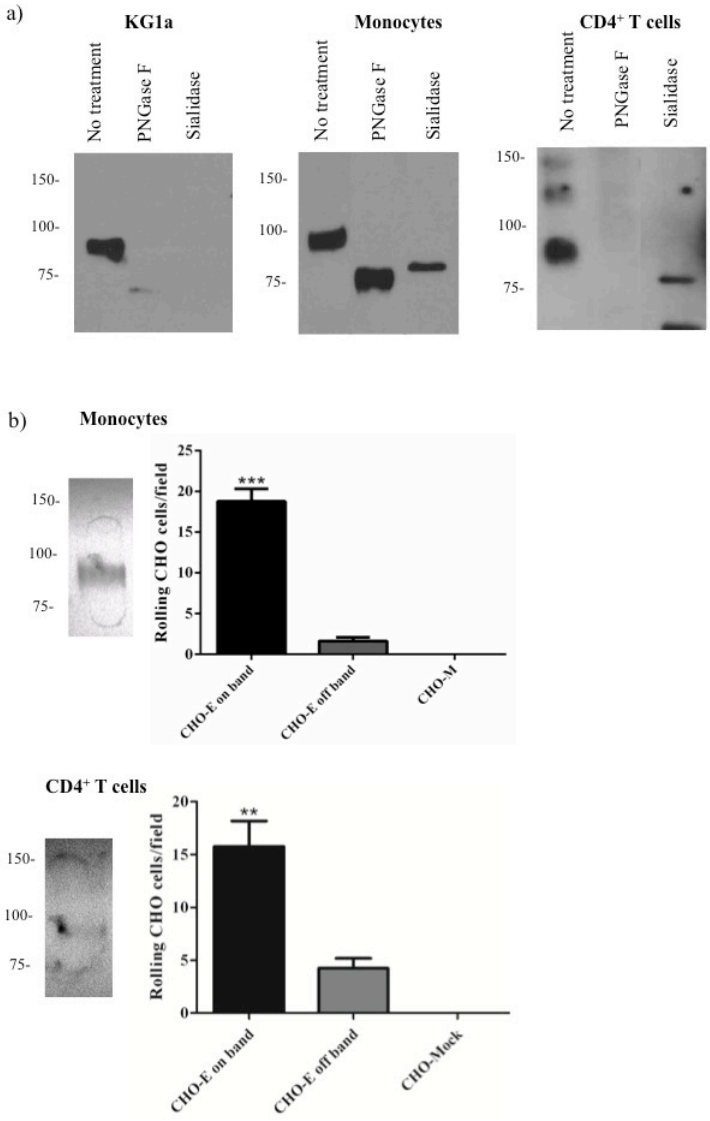

Figure 4. HCELL is a functional E-selectin ligand on human monocytes and CD4$^+$T-cells. (a) *CD44 expressed by monocytes displays sLe^x E-selectin binding determinants on O-glycans.* CD44 was immunoprecipitated from whole cell lysates of KG1a cells (control) (left panel), monocytes (middle panel) and CD4$^+$T-cells (right panel). CD44 immunoprecipitates were left untreated, or treated with PNGase-F or sialidase. Samples were resolved by SDS-PAGE, blotted and stained with E-Ig. In contrast to results obtained from PNGase-F digestion of HCELL of KG1a cells and CD4$^+$T-cells, the persistence of E-Ig staining after PNGase-F digestion of HCELL from monocytes shows that CD44 displays sLe^X *O*-glycans; results are representative of a minimum of three separate experiments. **(b)** *Blot rolling assay of HCELL from monocytes and CD4$^+$T-cells.* CD44 was immunoprecipitated from cell lysates of monocytes (upper) and CD4$^+$T-cells (lower). Immunoprecipitates were resolved by SDS-PAGE, blotted and stained with HECA-452 mAb. Monocyte and CD4$^+$T-cell lysates express the ~80-90kDa CD44 glycoform bearing sLe^X (i.e., HCELL), and functional E-selectin ligand activity was assessed by perfusion of E-selectin-transfected CHO cells (CHO-E) over blots at 0.17 dynes/cm^2. Non-specific adhesion was assessed by perfusing mock-transfected CHO cells (CHO-M) over the same regions of the blot. Data are mean ± SEM of 3 independent experiments.

B-cells express low levels of α(1,3)-Fucosyltransferases

To gain mechanistic insight into the observed absence of E-selectin ligand expression on B-cells, we evaluated, by RT-PCR, the principal glycosyltransferases and glycosidases that mediate display of sLe^X in monocytes and B-cells, which express the highest and lowest levels of E-selectin ligands, respectively (**Figure 5**). There are 6 fucosyltransferases - FTIII-VII and FTIX - that add α(1,3)-fucose to *N*-acetylglucosamine (GlcNac) within a type-2 lactosamine (i.e., galactose-β(1,4)-*N*-acetylglucosamine). FTIII/V-VII modify a sialylated-lactosamine chain to create sLe^X, whereas FTIV/IX only modify the core-lactosamine to make Le^X.[21] α(2,3)-sialylation to create sLe^X is programmed by three α(2,3)-sialyltransferases, ST3GalIII/IV/VI.[22-24] However, it is important to distinguish between sLe^X as displayed on relevant *O*- and *N*-linked glycans. On *O*-glycans, sLe^X is presented on a glycan-structure called "type-2 core extension". This core-2 structure is produced by core-2-synthase, C2GnT1, which catalyzes the addition of GlcNac to an acceptor glycan known as a "core-1" structure.[25] This process is abrogated by sialylation of the core-1 structure by ST3GalI or ST6GalNacII.[25,26] On *N*-glycans, the initiation of lactosamine chains requires addition of the first GlcNac residue, catalyzed by the enzyme α(1,3)-mannosyl glycoprotein 2-β-acetylglucosaminyltransferase (GlcNacT1/MGAT-1).[27] GDP-Fucose Transporter-1 (GDP-FucT1) is required for the uptake of GDP-fucose into Golgi-apparatus, where fucosylation of glycans and therefore formation of sLe^X takes palce.[28] In addition, fucosidase Fuc1[29] and sialidases Neu1 and Neu3[30,31] are responsible for cleaving fucose and sialic acid moieties from sLe^X glycan structures, respectively. The structural biology and enzyme mediators of sLe^X displayed on glycoproteins are summarized in **Figure 5a**.

As shown in **Figure 5b**, monocytes highly express all the α(1,3)-fucosyltransferases with the exception of FTV, whereas B-cells only express FTIV and FTVII. Interestingly, FTVII transcript levels were similar between B-cells and monocytes, whereas FTIV expression was increased in monocytes. Among the α(2,3)-sialyltransferases, ST3GalIII and ST3GalIV were expressed by both monocytes and B-cells, whereas ST3GalVI transcripts were only observed in monocytes. The transcript levels of glycosyltransferase ST6GalNacII and the glycosidases Neu3 and Fuc1 were found to be expressed at low levels in both monocytes and B-cells (**Figure 5b**), and Neu1 and GDP-FucT1 transcript levels were similar in both cell types. Surprisingly, C2GnT1 transcripts were detected at higher levels in B-cells than in monocytes. However, MGAT-1 levels are lower in B-cells than in monocytes, and, most importantly, B-

cells have high levels of ST3GalI transcripts whereas monocytes have very low levels. Thus, altogether, the "transcript signature" of B-cells reflects an enzyme expression pattern that would impede sLe^X display.

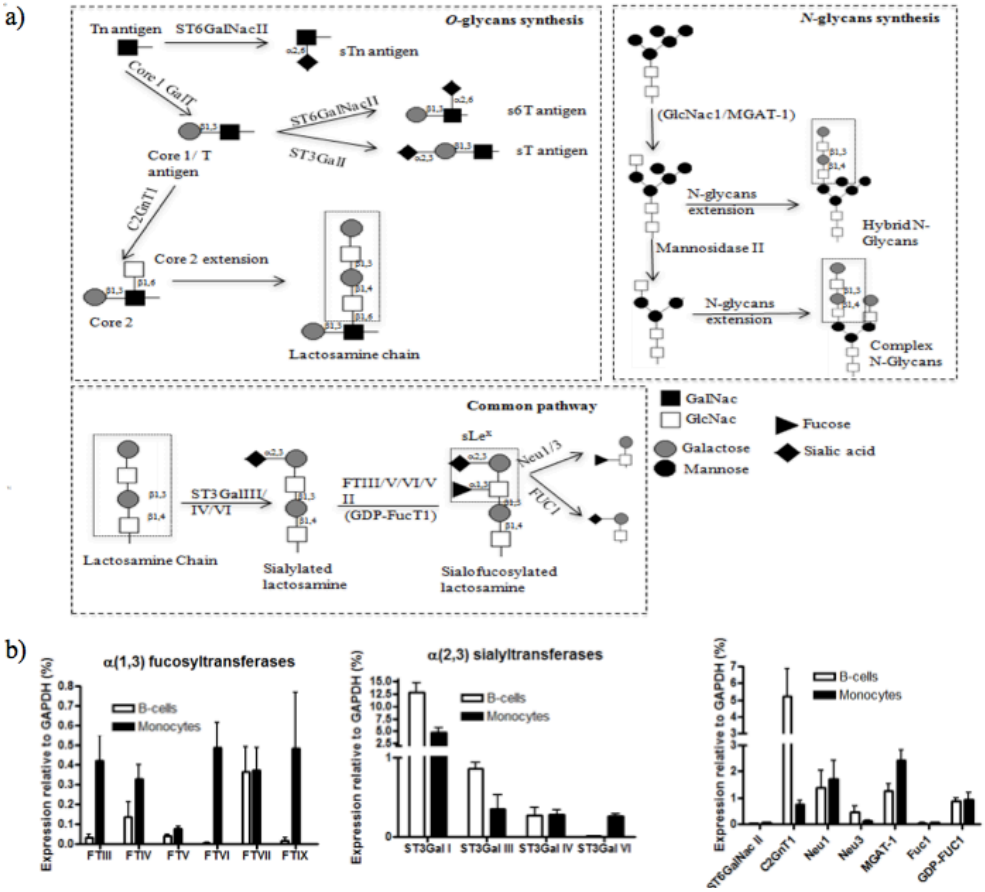

Figure 5. Analysis of glycosyltransferase gene expression on human monocytes and B-cells. (a) *Synthesis of O-linked and N-linked glycans carrying the* sLe^X *determinants.* *O*-glycan synthesis is initiated by an *N*-acetylgalactosaminyltransferase that transfers an *N*-acetylgalactosamine residue (GalNac) to a serine or threonine, forming the Tn antigen. Tn antigen can be sialylated by the activity of the α(2,6)-sialyltransferase ST6GalNac-II, yielding the sialyl-Tn antigen (sTn), which ceases further sugar modification. Alternatively, the transfer of galactose (Gal) to Tn antigen leads to formation of T antigen (core 1) by core 1 β(1,3)-galactosyltransferase (Core 1GalT). T antigen sialylation can be catalyzed by α(2,3)-sialyltransferase ST3GalI or α(2,6)-sialyltransferase ST6GalNac-II, forming sialyl-T (sT) or sialyl-6T (s6T) antigens, respectively. In absence of sialylation, core 1 can be modified by addition of *N*-acetylglucosamine (GlcNAc) by β(1,6)-*N*-acetylglucosaminyltransferase (C2GnT1), forming the "core-2" structure. Repetitive sequential, alternating additions of galactose and *N*-acetylglucosamine, respectively, create the lactosaminyl glycan chain that serves as an acceptor for terminal sialofucosylations to create sLe^X. On the substrate *N*-glycan $Man_5GlcNAc_2$ structure, biosynthesis of hybrid and complex glycans is initiated by the action of *N*-acetylglucosaminyltransferase-1 (GlcNAcT1/MGAT-1), which adds a GlcNac residue to the mannose present on the α(1-3)-arm of the core mannose. The resulting structure can either lose two mannose units off the α(1-6)-arm of the core mannose (by action of mannosidase II), leading to the formation of complex *N*-glycans, or not lose these mannoses, generating hybrid *N*-glycans. The sialyltransferases (ST3GalIII, IV and VI) terminate the elongation of both *O*- and *N*-Glycans by creating sialylated lactosamine chains that can be then fucosylated by the action of the fucosyltransferases FTIII, V, VI and VII, creating sLe^X. Neuraminidases 1 and 3 (Neu1, Neu3) and Fucosidase 1 (Fuc1) may then remove sialic acids or fucose moieties, respectively, down-regulating the expression of sLe^X epitopes. **(b)** *Real-time PCR analysis of glycosyltransferases involved in* sLe^X *biosynthesis in monocytes (black bars) and B-cells (white bars).* The mRNA expression of each glycosyltransferase was normalized to GAPDH.

B-cells exhibit a “transcript signature" of glycosyltransferases that favors a lower expression of sLe^X synthesis compared to monocytes. Experiments were performed with a minimum of six healthy donors.

FTVII exofucosylation enhances the expression of functional E-selectin ligands

Cell surface α(1,3)-exofucosylation can yield increased expression of E-selectin ligands,[5,32] but only if target cells display (unfucosylated) sialylated lactosaminyl glycans that can be converted to sLe^X determinants. To assess the extent to which α(1,3)-exofucosylation would enforce E-selectin ligand expression among the different PBMC subsets and the identity of relevant glycoprotein E-selectin ligands thus created, we treated cells with the enzyme fucosyltransferase VII (FTVII) in the presence of donor GDP-fucose. FTVII-treated monocytes showed dramatically increased expression of many HECA-452-reactive glycoproteins, including bands corresponding to CLA/CD43E, HCELL and the ~70kDa molecule (**Figure 6a**). To assess which of the protein scaffolds are preferentially targeted by exofucosylation, E-Ig-immunoprecipitates from lysates of buffer-treated and FTVII-treated monocytes were resolved by SDS-PAGE, and blotted with anti-PSGL-1, anti-CD44, and anti-CD43 antibodies. CD43E expression was dramatically increased following FTVII treatment, whereas the expression of CLA and HCELL increased to a lesser extent (**Figure 6a**). Interestingly, despite exhaustive immunoprecipitation of CD44 (**Figure 6b**), we observed persistent E-Ig-reactivity in the cleared lysates, indicating the presence of another sLe^X-decorated scaffold protein within the CD44 MW range.

For each lymphocyte subset, PSGL-1, CD43 and CD44 were sequentially immunoprecipitated from buffer-treated and FTVII-treated cells. For $CD4^+$T-cells, there was a minor increase in expression of CLA and CD43E after exofucosylation with a more marked increase in HCELL (**Figure 6c**). In contrast, in $CD8^+$T-cells, CLA and CD43E expression increased following FTVII treatment with only a minor increase in HCELL expression. However, in B-cells, exofucosylation engendered only CD43E, indicating that CD44 on B cells are not decorated with relevant acceptor sialyllactosaminyl glycans (**Figure 6c**).

To analyze the impact of exofucosylation on E-selectin binding of PBMC subsets, we compared the ability of buffer-treated and FTVII-treated cells to engage with E-selectin under physiological shear stress conditions. FTVII treatment resulted in significant increases in the number of tethering/rolling cells among all PBMC subsets (**Figure 6d**), yielding a 1.5- to 3-fold increase in the number of rolling cells for both monocytes and $CD4^+$T-cells, a 5- to 6-

fold increase in rolling of $CD8^+$T-cells, and a relatively modest but significant increase in rolling of B-cells. To assess E-selectin-dependent rolling/tethering specificity, PBMC subsets were sialidase-treated or HUVECs were pre-incubated with a function-blocking antibody against E-selectin; in each case, dramatic reductions in tethering/rolling was observed (**Figure 6d**).

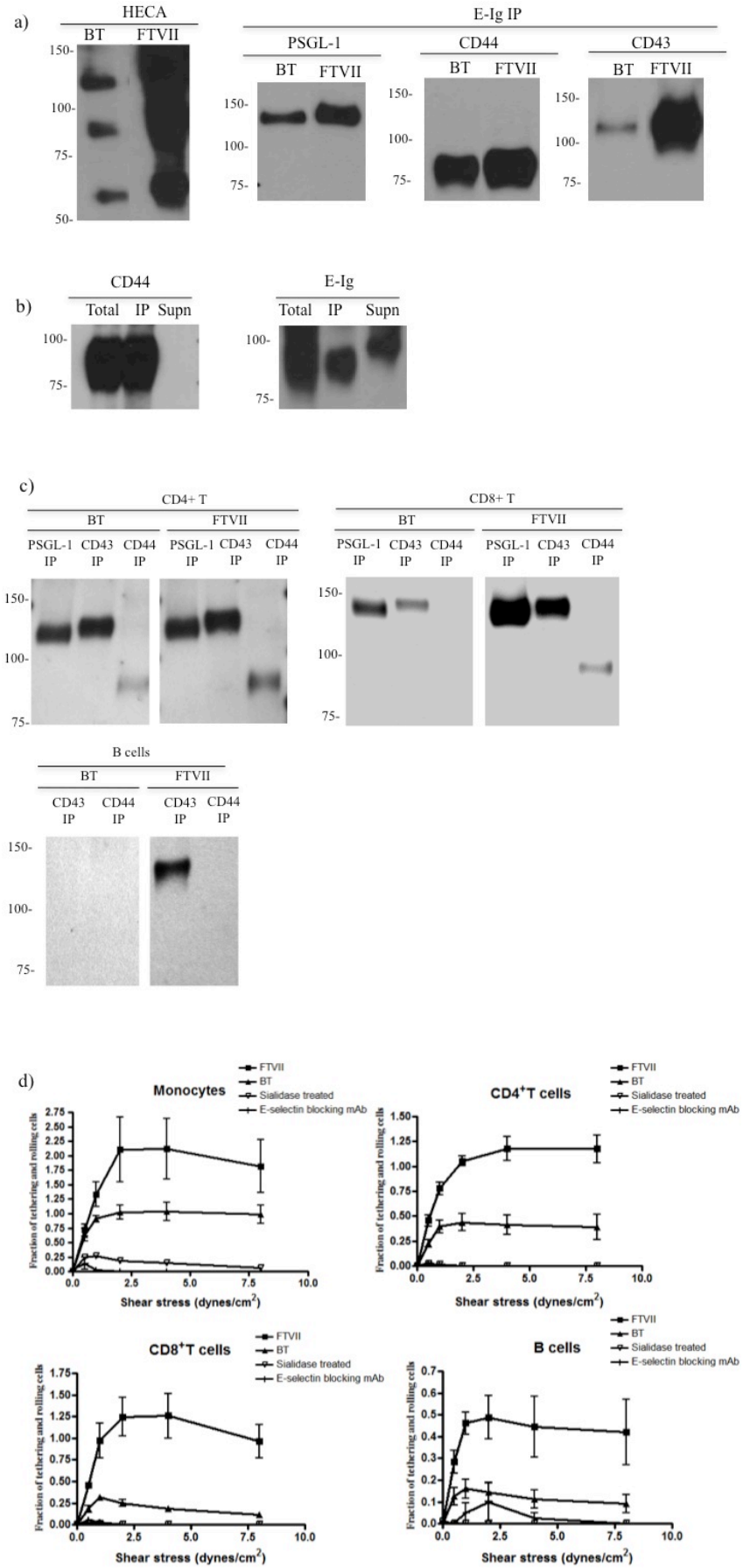
a)
HECA
BT
FTVII
150-
100-
75-
50-
E-Ig IP
PSGL-1
CD44
CD43
b)
CD44
E-Ig
Total
IP
Supn
c)
CD4+ T
CD8+ T
B cells
PSGL-1 IP
CD43 IP
CD44 IP
d)
Monocytes
CD4+T cells
CD8+T cells
B cells
FTVII
BT
Sialidase treated
E-selectin blocking mAb
Fraction of tethering and rolling cells
Shear stress (dynes/cm2)

Figure 6. E-selectin ligand expression on monocytes and lymphocytes is increased by cell surface α(1,3)-fucosylation. (a) *FTVII-mediated exofucosylation of monocytes creates E-selectin ligands on multiple cell surface glycoproteins.* Human monocytes were treated with fucosyltransferase VII (FTVII) or buffer alone (BT). Cell lysate was immunoprecipitated with E selectin-Ig (E-Ig IP), resolved by SDS-PAGE, and blotted with HECA-452, anti-PSGL-1, anti-CD43 or anti-CD44 antibodies. A marked increase in E-Ig reactivity on the CD43 scaffold was observed, with smaller increases of E-Ig reactivity on PSGL-1 and CD44. (**b)** *FTVII-mediated exofucosylation of monocytes creates a novel ~90-kDa E-selectin ligand.* CD44 immunoprecipitation was performed on cell lysate from FTVII-treated monocytes. Total cell lysate (Total), CD44 immunoprecipitate (IP), and cleared lysate (Supn) were resolved by SDS-PAGE and blotted with anti-CD44 mAb (left) or E-Ig (right). Following complete clearance of CD44, E-Ig-reactivity persists at ~90 kDa, indicating enforced expression of a presently unknown E-selectin ligand. (**c)** *FTVII-mediated exofucosylation of $CD4^+$T-cells, $CD8^+$T-cells, and B-cells variably converts PSGL-1, CD43 and/or CD44 to E-selectin ligands.* Human $CD4^+$T-cells, $CD8^+$T-cells, and B-cells were treated with fucosyltransferase VII (FTVII) or buffer alone (BT), and lysates were immunoprecipitated with PSGL-1, CD43, or CD44 mAbs. Immunoprecipitated products were resolved by SDS-PAGE and blotted with E-Ig. As shown in blot, exofucosylation of $CD4^+$T-cells increases E-selectin binding primarily on CD44, whereas exofucosylation increases E-selectin binding on all three scaffold proteins of $CD8^+$T-cells, and exclusively on CD43 of B-cells. (**d)** *FTVII treatment creates functional E-selectin ligands on monocytes and lymphocytes.* Human monocytes, $CD4^+$ and $CD8^+$T-cells and B-cells were treated with fucosyltransferase VII (FTVII) or buffer alone (BT), and were subsequently perfused into a parallel-plate flow chamber seeded with TNF-α-stimulated HUVEC at shear stresses of 0.5, 1, 2, 4, and 8 dynes/cm^2. All PBMC subsets exhibited significantly increased tethering/rolling after FTVII treatment. To control for E-selectin-dependent rolling specificity, PBMCs were pre-treated with sialidase, or HUVECs were preincubated with a function blocking mAb to E-selectin. Values are mean ± SEM of 3 independent experiments.

Discussion

In areas of tissue inflammation, those circulating leukocytes which express relevant molecular effectors of extravasation will accumulate at the affected site. In humans, the migration of circulating leukocytes into inflamed tissues is principally initiated by binding to E-selectin, which is induced by inflammatory cytokines within endothelial beds at all sites of inflammation.[33-35] To date, studies of the function and structure of leukocyte E-selectin ligands have focused largely on neutrophils. Our knowledge of the E-selectin ligands expressed among circulating mononuclear cells is grossly incomplete, and prior studies of mononuclear leukocyte E-selection ligands have concentrated principally on mouse cells.[4] Though sLe^X is an E-selectin binding determinant in both humans and mice, there are significant differences between human and murine leukocyte biology, and, more broadly, immunobiology.[36] Accordingly, an appropriate understanding of human leukocyte trafficking requires study of human cells, ideally, primary human cells. Here, we investigated the function and structural biology of the E-selectin ligands expressed on the major subsets of native human peripheral blood mononuclear cells (PBMCs): monocytes, B-cells, and $CD4^+$ and $CD8^+$T-cells.

Our findings reveal a previously unsuspected functional and structural diversity of E-selectin ligand expression among PBMC subsets. Using the parallel-plate flow chamber, we observed that among PBMCs, monocytes exhibit the most robust E-selectin binding activity under shear stress conditions, followed by $CD4^+$T-cells, but $CD8^+$T and B-cells exhibit little to no activity. These findings were consistent with flow cytometry results showing that monocytes exhibit the greatest reactivity to E-selectin-Ig (E-Ig) and to HECA-452, followed, in decreasing order, by $CD4^+$T-cells, $CD8^+$T-cells, and B-cells. The strong correlation between HECA-452 staining and both E-Ig-reactivity and E-selectin binding under shear stress is reflective of the fact that sLe^X is the predominant E-selectin binding determinant expressed on PBMCs. Moreover, the major carriers of this determinant are glycoproteins rather than glycolipids, as shown by the significant abrogation of HECA-452 reactivity upon cell surface protein digestion with bromelain.

To identify the specific E-selectin ligands expressed by PBMCs, Western blotting was conducted using E-Ig as a probe. In monocyte lysates, we observed three distinct E-Ig-reactive bands under reduced SDS-PAGE conditions: the upper band (~120-130 kDa) is comprised of CD43E and the monomer form of CLA, the middle band (~80-90 kDa) is HCELL, and the lower band (~70 kDa) is a currently-unidentified E-selectin ligand unrelated to the structure known as MPO-EL.[16] Our dataset is the first to describe expression of CD43E on monocytes. CD43 has previously been described to function as an E-selectin ligand on both human and murine T-cells[14,37,38] and HSPCs,[5] and on murine neutrophils.[39]

In western blots of $CD4^+$T-cell lysates, we observed two distinct E-Ig-reactive bands at ~120-130 kDa and ~80-90 kDa, while $CD8^+$T-cells express only one E-Ig-reactive band at ~120-130 kDa and B cells do not express any E-selectin-reactive glycoproteins. As with monocytes, the ~120-130kDa band consists of CD43E and the monomer form of CLA, both of which are natively expressed by $CD4^+$ and $CD8^+$T-cells. In addition, we identified HCELL as the ~80-90 kDa E-selectin ligand expressed by $CD4^+$T-cells.

Our data show, for the first time, that HCELL, a human E-selectin ligand previously recognized on native HSPCs[5,15], can be expressed on mature human leukocytes. However, HCELL of human HSPCs and of $CD4^+$T-cells expresses its E-selectin-reactive sialofucosylated determinants on *N*-glycans, whereas the HCELL glycoform of monocytes displays these determinants on *O*-glycans. By western blot, the E-Ig-reactivity of HCELL is higher on monocytes than on $CD4^+$T-cells, at a level similar to that observed on HSPCs, and

blot rolling assays indicate that HCELL is a functional E-selectin ligand in both monocytes and $CD4^+$T-cells. HCELL is the most potent E-selectin ligand expressed on human cells[1,5], and HCELL expression on human monocytes and $CD4^+$T-cells is consistent with the higher capacity of these cells to undertake tethering/rolling adhesive interactions on TNF-α-stimulated endothelium under shear stress (**Figure 1a**). Importantly, we found that human PBMCs do not express the glycoprotein known as E-selectin ligand-1 (ESL-1) that functions as the most potent E-selectin ligand on mouse leukocytes.[40] Collectively, this discrepancy and the observed differences in HCELL expression among PBMCs underscore the key fact that there is considerable species-specific and cell-specific variability in the structural biology of E-selectin ligands.

To gain mechanistic insight into cell type-specific differences in E-selectin ligand expression, we performed quantitative PCR studies of relevant glycosyltransferases directing sLe^X synthesis on B-cells and monocytes, and performed α(1,3)-exofucosylation of the different PBMC subsets to probe the breadth and relative level of expression of sialyllactosaminyl glycan acceptors. Human B-cells have low expression of fucosyltransferase genes, but broadly express transcripts encoding the α(2,3)-sialyltransferases necessary for the production of sialyllactosaminyl glycans. Moreover, compared to monocytes, B-cells expressed higher and lower levels of ST3GalI and MGAT-1, respectively. A higher expression of ST3GalI is associated with the formation of the sialyl-T (sT) antigen, blocking core-2 formation and thereby down-regulating sLe^X displayed on *O*-glycans.[41] The lower expression of MGAT-1 results in fewer lactosaminyl glycans on *N*-glycans.[42] Collectively, this pattern of glycosyltransferase gene expression, in addition to the relatively low expression of scaffold proteins for sLe^X decorations, accounts for the paucity of E-selectin ligand expression in B-cells. However, among all the different PBMC subsets, α(1,3)-exofucosylation markedly increased E-selectin binding activity, indicating that each subset displays acceptor terminal sialylated type-2 lactosamine units that can be extrinsically fucosylated to create the sLe^X determinant. Notably, the protein scaffolds preferentially targeted by exofucosylation in the various PBMC subsets were strikingly different. The fact that monocytes, $CD4^+$ and $CD8^+$T-cells express equivalently high levels of PSGL-1, CD44, and CD43 - and yet these proteins are differentially modified with sialofucosylated lattice - indicates that there is cell-specific sorting/segregation of protein scaffolds that serve as acceptors for glycan modification within the Golgi-apparatus. Paradoxically, although B-cells express much more CD44 than CD43, exofucosylation yielded expression of only

CD43E, indicating that CD43 is the predominant carrier of sialyllactosaminyl glycans on B-cell surface (**Figure 6c**). Commensurately, although exofucosylation upregulated E-selectin binding activity on all PBMC subsets (**Figure 6d**), B-cells had the lowest capacity to engage E-selectin under hemodynamic shear conditions.

The orchestration of immunity critically depends on the efficient migration of relevant immune effector cells. The ability of circulating leukocytes to adhere to vascular endothelium is fundamental to initiating and propagating any immune response, and, as such, the distinct repertoires of adhesion molecules expressed on the surface of PBMCs are tightly linked to the roles of such cells in immunity. This study is the first comprehensive analysis of the structures that serve as E-selectin ligands on relevant subsets of primary human PBMCs. The observed distinct differences in E-selectin ligand expression among circulating human lymphocytes and monocytes provide key insights into their native ability for migration in steady-state into skin or marrow (where E-selectin is constitutively expressed), and in response to inflammation and/or tissue injury (where E-selectin expression is upregulated).[43] Notably, our results provide first evidence that human monocytes exhibit remarkably high E-selectin ligand activity which is conferred by expression of multiple E-selectin ligands including HCELL, a highly potent E-selectin ligand. The high E-selectin binding activity of monocytes offers mechanistic insight on the conspicuous propensity of these cells to adhere to inflamed vascular endothelium, a process that underlies several vascular inflammatory diseases, including atherosclerosis.[44] Moreover, the prominent E-selectin ligand activity mediates monocyte tropism to inflamed endothelial beds thereby licensing immunosurveillance appropriate to the role(s) of these cells as key sentinels of host defense and effectors of antigen recognition and immunoregulation.

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Authorship Contributions and Disclosure of Conflicts of Interest

The study was conceived by R.S., who designed and supervised all research, funded the research and wrote the manuscript. P.V. designed and supervised research, funded the research and wrote the manuscript. M.S., R.K.F.F., C.B.D. designed and performed research, collected and analyzed data, and wrote the manuscript. According to the National Institutes of Health policies and procedures, the Brigham & Women's Hospital has assigned intellectual property rights regarding HCELL to the inventor (R.S.), who may benefit financially if the technology is licensed. R.S.'s ownership interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policy. All other authors declare no competing financial interests.

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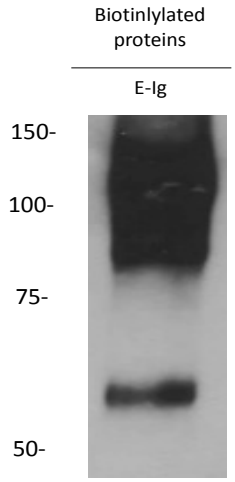
Supplementary Figures

Protein	Forward Primer (5’–3’)	Reverse Primer (5’–3’)	Reference
FTIII	GCCGACCGCAAGGTGTAC	TGACTTAGGGTTGGACATGATATCC	45
FTIV	GGGTTTGGATGAACTTCGAGTCG	GGTAGCCATAAGGCACAAAGACG	OriGene
FTV	ACCTGAGCTACTTTCACTGGCG	TCAGGTGAACCAAGCCGCTATG	OriGene
FTVI	AATGGGTCCCGCTTCCCAGACAG	GCGTCCGTACACGTCCACCTTG	46
FTVII	TCCGCGTGCGACTGTTC	GTGTGGGTAGCGGTCACAGA	45
FTIX	TCCCATGCAGTTCTGATCCAT	GAAGGGTGGCCTAGCTTGCT	45
GlcNacT-1/MGAT-1	CCTATGACCGAGATTTCCTCGC	TGAAGCTGTCCCTGCCCGTATA	OriGene
C2GnT1	AACCCCTTAGTAAAGAAGAGGCG	AGCAGCCTGTCAAGCATTTCA	PrimerBank
GDP-Fucose Transporter 1	CTGCCTCAAGTACGTCGGTG	CCGATGATGATACCGCAGGTG	47
ST3GalI	AAGAGGACCCTGAAAGTGCTC	CTCCAGGACCATCTGCTTGG	PrimerBank
ST3GalIII	GCCTGCTGAATTAGCCACCAA	GCCCACTTGCGAAAGGAGT	PrimerBank
ST3GalIV	CTTCCTGCGGCTTGAGGATTA	CTCACTCCCCTTGGTCCCATA	PrimerBank
ST3GalVI	ACTGCATTGCATATTATGGGGAA	TGGCTTTGATAAACAAGGCTGG	OriGene
ST6GalNAcII	ACTTCCGTGGCCTGTTCAATC	GGCGATGACTTGGTGAGAGAG	PrimerBank
Neu1	GGAGGCTGTAGGGTTTGGG	CACCAGACCGAAGTCGTTCT	PrimerBank
Neu3	AAGTGACAACATGCTCCTTCAA	TCTCCTCGTAGAACGCTTCTC	PrimerBank
Fucosidase1	AGTCACCCTGTTGCCTATGG	TTTGGCGCTTTTAGATTGCT	48

Supplemental Table S1
Primers used in Real-time PCR analysis of glycosyltransferases genes involved in sLe^X synthesis.[45-48]



Supplemental Fig. S1
E-selectin-reactive 70kDa unidentified glycoprotein in monocytes is a cell surface protein. Monocyte surface was labeled with biotin derivate products and lysed. Biotinylated proteins were collected on streptavidin beads, SDS/PAGE resolved, blotted, and stained with E-Ig. Three E-Ig reactive bands were observed, including the 70kDa unidentified glycoprotein, confirming its expression on the cell surface.

Chapter 4

Functional Diversity of CD44 in Culture-Expanded Human Monocyte-derived Dendritic Cells

Paper II- ***submitted to blood***

TITLE

Functional Diversity of CD44 in Culture-Expanded Human Monocyte-derived Dendritic Cells

RUNNING TITLE

CD44 pleiotropism in cultured human dendritic cells

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KEY POINTS

(1) **HCELL, a CD44 glycoform that potently binds E-selectin, is expressed on human CD14-selected monocyte-derived DCs (CD14-S mo-DCs).**
(2) **CD44 engagement on CD14-S mo-DCs triggers VLA-4-dependent adhesiveness, yielding transendothelial migration in absence of chemokine input.**

Abstract

The ability of circulating dendritic cells (DCs) to extravasate at inflammatory sites is critical to immunoregulation. Here, we examined the adhesion molecules mediating transendothelial migration (TEM) of culture-expanded human monocyte derived-DCs (mo-DCs) on TNFα-stimulated HUVEC monolayers. We observed that in absence of applied chemokine gradients, mo-DCs undertake TEM and that mo-DCs generated by monocyte enrichment via CD14-selection (CD14-S) display 2-fold greater TEM than those generated following plastic adherence-selection (PA-S). Binding assays using function-blocking mAb and substrate adhesion molecules showed that the augmented TEM of CD14-S mo-DC is E-selectin- and VLA-4-dependent, and that engagement of E-selectin ligands induces VLA-4 activation on CD14-S mo-DCs but not on PA-S mo-DCs. Biochemical studies showed that E-selectin binding glycoforms of PSGL-1 (i.e., CLA) and CD44 (i.e., HCELL) were both expressed on CD14-S mo-DCs, but only CLA was found on PA-S mo-DCs. Exoglycosylation of CD14-S mo-DCs using α-(1,3)-fucosyltransferase significantly increased expression of CLA and HCELL, and these glycoengineered cells exhibited increased TEM. To evaluate how engagement of CD44 and PSGL-1 each influence TEM of mo-DCs, cells were treated with their ligands hyaluronic acid or P-selectin, respectively, prior to incubation with stimulated HUVECs. Ligation of CD44 on CD14-S mo-DCs, but not CD44 on PA-S mo-DCs, triggered VLA-4 activation and TEM, whereas PSGL-1 ligation had no effect on VLA-4 function. These findings highlight structural and functional pleiotropism of CD44 in priming TEM of culture-expanded mo-DCs, and suggest that strategies to enforce HCELL expression could boost CD14-S mo-DC recruitment at inflammatory sites by augmenting E-selectin-driven, CD44-dependent VLA-4 activation.

Introduction

Dendritic cells (DCs) are leukocytes specialized to capture and present antigens. DCs are located within tissues throughout the body, where they serve as sentinels of host defense, orchestrating immune responses by activating naïve and memory T cells.[1] Though DCs can prime T cell immunoreactivity within tissues *in situ*,[2,3] they typically exit tissues via afferent lymphatics, thereafter accumulating in lymph nodes wherein they induce proliferation of antigen-responsive T cells. As such, the extravasation of DC at inflammatory sites enables antigen presentation and T cell activation.[4] Previous studies have indicated that in vitro conditions for generating DCs can negatively affect tissue migration in vivo, thus markedly lessening their efficacy in adoptive DC-based immunotherapeutics.[5,6]

Extravasation of blood-borne cells involves a multistep cascade of events initiated by hemodynamic shear-resistant adhesion of cells to target endothelium. This important function is mediated most efficiently by engagement of selectins with sialofucosylated glycans, prototypically sialyl Lewis X (sLe^x; NeuAc-α(2,3)-Gal-β(1,4)-[Fuc-α(1,3)]GlcNAc-R), displayed on cell surface protein or lipid scaffolds.[7] The vascular selectins are comprised of E- and P-selectin, which are inducible by inflammatory cytokines.[8,9] Sialofucosylated ligands for vascular selectins serve as tissue "homing receptors" on circulating cells, and mediate tethering and rolling of the cells on the endothelium (Step 1). The cells then undergo "Step 2" activation of integrin adhesiveness, typically via chemokine receptor signaling, leading to firm adhesion on the endothelium (Step 3), and then transendothelial migration (TEM) (Step 4).[7] Importantly, the principal inflammatory cytokines, tumor necrosis factor–α (TNFα) and interleukin (IL)-1β, upregulate both E- and P-selectin expression in murine endothelial cells, but the promoter elements responsive to TNFα and IL-1β in the human P-selectin gene are absent.[10] Thus, in humans, E-selectin receptor/ligand interactions play a predominant role in recruitment of cells to inflammatory sites, prompting development of strategies to enforce E-selectin ligand expression on relevant human cells to optimize their utility in adoptive immunotherapeutics.

The molecular effectors mediating DC adherence to endothelium and consequent TEM have not been fully elucidated. To date, P-selectin glycoprotein ligand-1 (PSGL-1) is the only sLe^x scaffold reported to be expressed on human DCs, and PSGL-1 is the principal P-selectin counterreceptor in human mo-DCs.[11-13] Though a heavily sialofucosylated glycoform of PSGL-1 that binds E-selectin (known as "Cutaneous Lymphocyte Antigen" (CLA)) has been

described on DCs,[11] our knowledge of the breadth and function of human mo-DC glycoconjugate counterreceptors for E-selectin is incomplete. In humans, hematopoietic stem cells express a CD44 glycoform known as Hematopoietic Cell E-selectin/ L-selectin Ligand (HCELL),[14,15] which serves as a potent E-selectin ligand. CD44 expression is characteristic of human DCs and is known to be critical for DC lodgment within T cell zones of lymph nodes and formation of immunological synapses,[16,17] but its role in mediating recruitment of blood-borne DC into tissues has not been evaluated.

In this study, we examined TEM of human blood monocyte-derived DCs (mo-DCs) on primary cultures of human umbilical vein endothelial cells (HUVECs) treated with TNFα to mimic inflammation-related endothelial expression of E-selectin and vascular cell adhesion molecule-1 ((VCAM-1), a ligand for integrin VLA-4). We observed that mo-DCs generated from monocytes enriched using CD14-bead selection (CD14-S mo-DCs) or plastic adherence selection (PA-S mo-DCs) can each execute TEM in absence of applied chemokine gradients, but CD14-S mo-DCs display much greater TEM than do PA-S cells. TEM is dependent on engagement of mo-DC E-selectin ligands, and, notably, both HCELL and CLA are expressed by CD14-S mo-DCs whereas only CLA is expressed on PA-S mo-DCs. Engagement of CD44 on CD14-S mo-DCs, but not CD44 on PA-S mo-DCs, directly activates VLA-4 thereby programming TEM on TNFα stimulated HUVECs, and enforced HCELL expression by α-(1,3)-exofucosylation of CD14-S mo-DC significantly increases TEM. Collectively, these findings unveil a previously unrecognized structural and functional versatility of CD44 on culture-expanded human DCs, and suggest that glycosylation of CD44 to engender HCELL expression may be exploited to promote DC migration to sites of inflammation.

Material and Methods

Cell isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy donors obtained by venipuncture, under protocols approved by the Institutional Review Board of Brigham & Women's Hospital, with Informed Consent provided as per the Declaration of Helsinki. Blood monocytes were enriched using anti-CD14 mAb-coated magnetic beads

(Miltenyi Biotech) (CD14-S) or by plastic adherence (PA-S), and cultured as described.[18,19] After 5 days, mo-DCs were matured with TNFα (25 ng/mL, R&D Systems) for 2 or 3 days (as specified), or left untreated (immature DCs). For activation, mo-DCs were treated with 200 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma) in RPMI media for 30 min at 37ºC. HUVECs were obtained from the BWH Pathology Core facility and cultured as described.[13,20]

Antibody reagents and flow cytometry

Cell surface staining of mo-DCs for flow cytometry was performed as described previously[13,18] using commercially-available mouse E-selectin-human Fc Ig chimera (E-Ig) and antibody reagents directed to sLe^x, CD14, CD44, PSGL-1, MHC-II, CD86, and to resting and activation-dependent epitopes of VLA-4 and LFA-1 (for full description, see Supplemental Methods).

Blot Rolling Assay

Blot rolling assays to detect E-selectin ligands on PVDF membrane blots of cell lysate proteins resolved by SDS-PAGE were performed as previously described,[21] (see Supplemental Methods for details).

Isolation of RNA and Real-Time PCR

Total RNA was extracted and reverse transcribed as described.[18] Real-Time PCR (RT-PCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primers were selected from PrimerBank[22] (supplemental Table S1) and gene expression was normalized to *GAPDH* (endogenous reference) expression. The normalized mRNA expression was computed as a permillage fraction (‰), which means the proportion of target mRNA per thousand of the reference *GAPDH* mRNA expression, calculated by using the $2^{-\Delta CT}\times 1000$ formula.[23,24] Amplification specificity was confirmed by melting curve analysis.

Transendothelial migration (TEM) assays

For TEM analysis under non-shear conditions, mo-DCs (4×10^5 cells/ well) were labeled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) and placed in a transwell chamber containing HUVEC monolayers (cultured on 8 μm pore membrane, Millipore); to induce HUVEC E-selectin and VCAM-1 expression, monolayers were exposed to TNFα (40 ng/mL) for 6 h ("stimulated" HUVECs). All assays were performed under serum- and chemokine-free conditions at 37°C for 12 h, and cell migration to the underside of transwell membrane was quantified by fluorescence microscopy. Controls consisted of evaluation of TEM on unstimulated HUVEC monolayers, and on stimulated HUVECs treated with function blocking anti-human E-selectin or isotype control mAb (20 μg/mL, 30 min). In some experiments, mo-DCs were treated with function blocking anti-VLA-4 mAb HP2/1 (Millipore) or anti-LFA-1 mAb 24[25] (20 μg/mL, 30 min) or pertussis toxin (PTX, Sigma) (250 ng/mL, 2 h).

For TEM analysis under hemodynamic fluid shear conditions, HUVECs were cultured on the underside of a transwell insert, stimulated with TNFα (as above), and placed into a flow chamber mounted on an inverted microscope, as previously described.[20] Mo-DCs (4×10^6 cells/mL) were perfused at 37°C over HUVECs at an initial shear rate of 0.5 dynes/cm^2 with subsequently increasing shear stress stepwise every 30 seconds until 7.5 dynes/ cm^2. The number of tethering and rolling mo-DCs, per cm^2 HUVEC area was quantified during each 30 second segment and the number of cells that became firmly adherent was recorded.[20] The average rolling velocity, a measure of selectin binding strength, was evaluated at 2.0 dyn/cm^2, as the displacement of the centroid of the cell over 1 sec intervals. All experiments were performed and video-recorded for analysis.

CS-1 fragment binding assay following E-selectin and hyaluronic acid engagement

Mo-DCs were placed in culture plates seeded with CHO-E or with mock transfectants (CHO-mock), or coated with E-Ig or high molecular weight hyaluronic acid (HA), as detailed in the Supplemental Methods. Binding of mo-DCs to segment-1 peptide (CS1) was then assessed (see Supplemental Methods for full description).

Western blotting and immunoprecipitation

Immunoprecipitation of CD44, PSGL-1, and E-selectin ligands (collected using E-Ig as probe) was conducted as described in the Supplemental Methods. Cell lysate proteins or immunoprecipitated proteins were resolved on reducing SDS-PAGE gels and electrotransferred to PVDF membranes. E-Ig staining was done using a 3-step protocol (E-Ig immunostaining, followed by rat anti-mouse CD62E mAb (R&D Systems), then followed by horseradish peroxidise (HRP)-conjugated anti-rat IgG). Specificity for E-Ig binding was confirmed by loss of E-Ig reactivity in presence of EDTA and following sialidase treatment (to remove sialic acid from sLe^x).

Fucosyltransferase VI treatment

Cells were treated as previously described[26] with 60 mU/mL FTVI[27], and effectiveness of α(,3)-exofucosylation was assessed by flow cytometry and western blot analysis of sLe^x expression and E-Ig reactivity (for details, see Supplemental Methods).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical significance was determined using the GraphPad PRISM software version 6.00 (San Diego, CA). Differences between means were determined by one-way analysis of variance. Multiple comparisons were performed post-hoc by the Tukey *t* test. Statistical significance was defined as $P \leq 0.05$.

Results

CD14-S mo-DCs display markedly higher TEM than PA-S mo-DCs on stimulated HUVEC monolayers

To assess whether monocyte enrichment methods affect endothelial adhesive interactions of differentiated mo-DCs, we compared the ability of CD14-S and PA-S mo-DCs to execute TEM on unstimulated HUVECs and HUVECs stimulated with TNFα to up-regulate E-selectin and VCAM-1 expression.[28] While both types of mo-DCs have higher TEM on stimulated compared to unstimulated HUVECs, CD14-S mo-DCs undertake markedly more TEM on stimulated HUVECs compared to PA-S mo-DCs (Figure 1A); this augmented TEM was abrogated by function blocking antibodies against E-selectin and by sialidase treatment of mo-DCs, highlighting a key role for E-selectin receptor/ligand interactions in the extravasation process (Figure 1A). Moreover, the heightened TEM of CD14-S mo-DCs was blocked by function-blocking mAb to the VCAM-1 ligand, VLA-4 (i.e., α4β1 or CD49d/CD29) but not to the ICAM-1 ligand LFA-1 (αLβ2, CD11a/CD18) (Figure 1A), indicating that the observed TEM on stimulated HUVEC requires engagement of both E-selectin/E-selectin ligands and VLA-4/VCAM-1. Furthermore, pretreatment with pertussis toxin (PTX), a specific inhibitor of Gαi-dependent signaling, inhibited TEM of both types of mo-DC (Figure 1A), indicating that G-protein-mediated signaling is requisite for mo-DC transmigration.

In human mesenchymal stem cells and lymphoid cells,[26,27,29] it has been reported that engagement of E-selectin ligands directly up-regulates VLA-4 integrin-mediated binding in the absence of chemokine input. We thus sought to examine whether β1 integrin activation occurs following engagement of E-selectin ligands on CD14-S and PA-S mo-DCs. To assess VLA-4 function, we tested mo-DC adhesiveness to CS-1 fibronectin fragment, binding to which depends on activation of VLA-4. Engagement of CD14-S mo-DCs on immobilized E-selectin significantly increased adhesiveness of these cells to CS-1 fibronectin fragment (Figure 1B). CS-1 adhesiveness was significantly reduced by function-blocking mAb against E-selectin, confirming the contribution of E-selectin engagement in priming VLA-4 binding to CS-1 (Figure 1B). However, engagement of PA-S mo-DCs on E-selectin did not induce significant mo-DC adherence to CS-1 fibronectin (Figure 1B), which was consistent with their reduced TEM ability on stimulated HUVECs. Staining with mAb directed to activation epitope of VLA-4 β1-chain (CD29) was observed after engagement of CD14-S mo-DCs on

immobilized E-selectin and was not observed on PA-S cells, consistent with lack of fibronectin binding by these cells (Figure 1C). Activation of the β2-integrin LFA-1 (CD11/CD18) after engagement on immobilized E-selectin was not observed on CD14-S mo-DCs (Supplemental Figure S1). Collectively, these data show that engagement of E-selectin ligands on human CD14-S mo-DCs, but not PA-S mo-DCs, leads directly to activation of VLA-4 and subsequent transmigration.

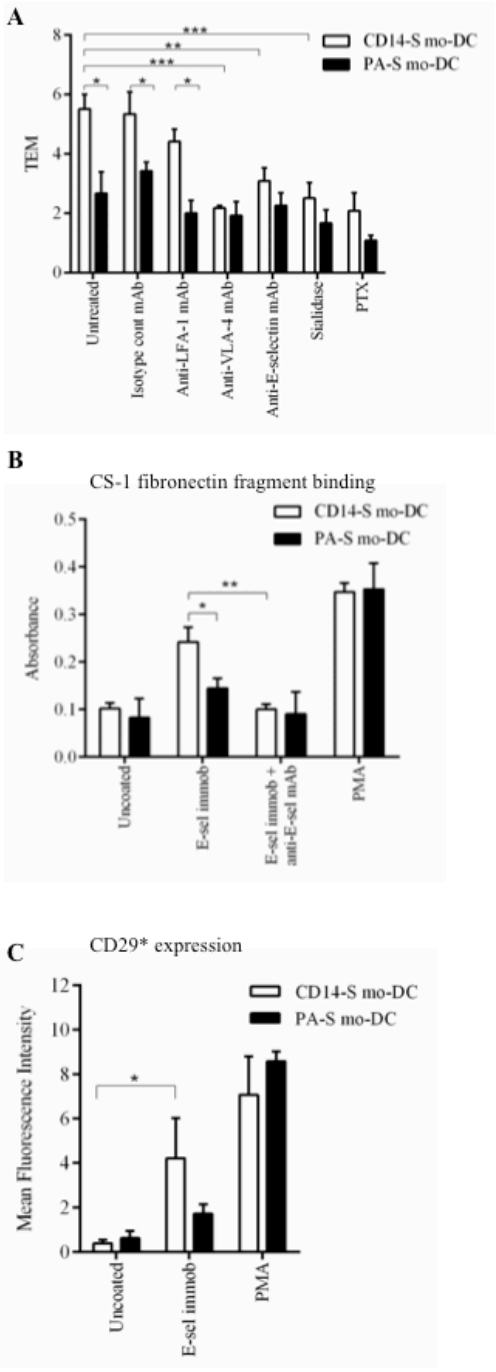

Figure 1. Transendothelial migration of mo-DCs is dependent on engagement of E-selectin ligands and VLA-4. (A) *Transendothelial migration (TEM) assay of CD14-S and PA-S mo-DCs on HUVEC monolayers.* The relative TEM value is the ratio of mo-DCs transmigrated on TNFα-stimulated HUVECs compared with mo-

DCs transmigrated on non-stimulated HUVECs. TEM was assessed using mo-DCs (untreated), mo-DCs preincubated with isotype control or with function blocking anti-LFA-1 mAb or with function blocking anti-VLA-4 mAb, HUVECs preincubated with function blocking anti-E-selectin mAb, and mo-DCs treated with sialidase or pertussis toxin (PTX). **(B)** *Adhesiveness to CS-1 fibronectin fragment of CD14-S or PA-S mo-DCs.* Cells were first incubated on uncoated or E-selectin-coated plates and then tested for binding to CS-1 peptide coated plates. Mo-DCs were stained with crystal violet and adherence was quantified by measuring light absorbance at 595 nm. Function blocking anti-E-selectin mAb was used as negative control and PMA treatment used as a positive control. **(C)** *Flow cytometry analysis of activated β1-integrin expression by CD14-S and PA-S mo-DCs*. Mo-DCs were incubated on uncoated plates or on E-selectin-coated plates, or cells were stimulated with PMA, and then stained with mAb HUTS-21 that identifies an activation-dependent epitope of β1-integrin (CD29*). Graph values represent the mean fluorescence intensity of HUTS-21 staining (n= 3, mean ± SD) subtracted from the values obtained using isotype control.

HCELL is expressed by CD14-S mo-DCs

To identify the molecular effectors of mo-DC TEM, we compared expression of E-selectin ligands and of VLA-4 in both CD14-S and PA-S mo-DCs. By flow cytometry, expression of VLA-4, PSGL-1, and CD44 was equivalent between CD14-S and PA-S mo-DCs (Figure 2A), and, though HECA-452 reactivity (i.e., sLe^x expression) was similar on both types of mo-DCs, E-Ig reactivity was slightly higher in CD14-S mo-DC (Figure 2A). However, western blot analysis of reduced SDS-PAGE gels revealed pronounced differences in E-Ig-reactive bands between CD14-S and PA-S mo-DCs (Figure 2B). For both types of mo-DCs, E-Ig stained a ~120-130 kDa band, characteristic of the PSGL-1 glycovariant, CLA (Figure 2B). E-selectin-reactive proteins (i.e., E-Ig immunoprecipitants) of CD14-S and PA-S mo-DCs, showed equivalent reactivity with PSGL-1 (Figure 2C) thus confirming the expression of CLA by both types of mo-DCs. Importantly, only CD14-S mo-DCs showed an additional E-Ig reactive ~80-90 kDa band (Figure 2B), a migration pattern consistent with that of the CD44 glycoform HCELL. Consistently, CD44 immunoprecipitated from CD14-S mo-DCs, but not from PA-S mo-DCs, showed reactivity with E-Ig, confirming that the E-Ig reactive ~80-90 kDa protein on CD14-S mo-DCs is HCELL (Figure 2D). Moreover, in blot rolling assays, CD44 immunoprecipitated from CD14-S mo-DC lysates supported CHO-E rolling interactions under physiologic shear conditions (Figure 2E).

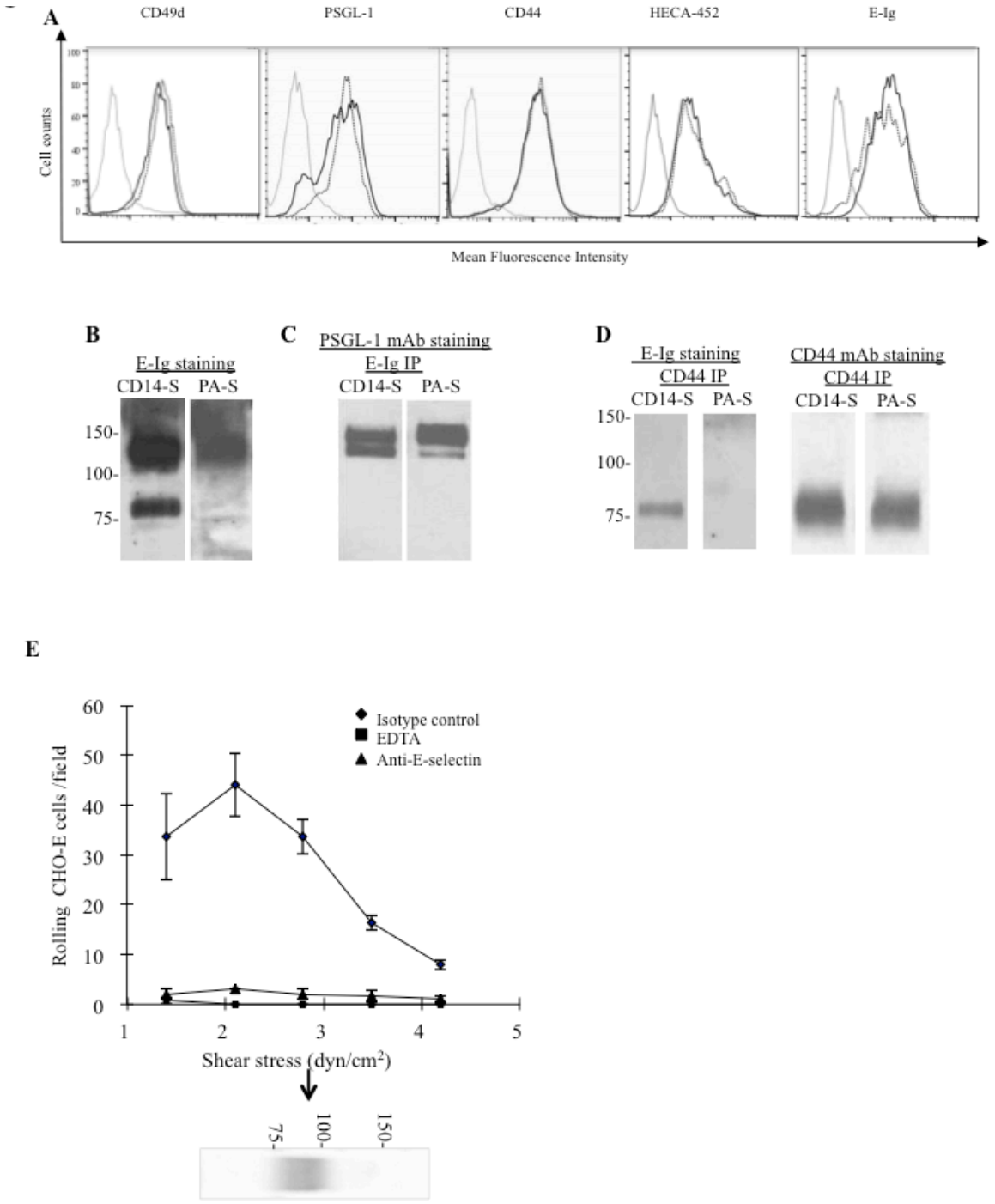

Figure 2: Analysis of VLA-4 and E-selectin ligand expression on mo-DCs. (A) *Representative flow cytometry analysis of mo-DCs staining using mAbs to CD49d (VLA-4), PSGL-1 and CD44, and HECA-452 mAb and E-Ig.* Grey lines represent isotype control (or, for E-Ig, staining in the absence of Ca^{2+}), dotted black line represents PA-S mo-DCs and solid black lines are CD14-S mo-DCs. **(B-D)** *Analysis of glycoprotein E-selectin ligands expressed by mo-DCs:* **(B)** Equivalent amounts of cell lysates of CD14-S and PA-S mo-DCs were resolved by SDS-PAGE electrophoresis, and immunoblotted with E-Ig chimera. Two E-selectin-reactive bands were visible in lysates of CD14-S mo-DCs (~130 kD and ~80 kD), whereas only one band (~130 kD) was reactive on PA-S mo-DCs; **(C)** Equivalent amounts of cell lysates of CD14-S and PA-S mo-DCs were immunoprecipitated with E-Ig, and immunoprecipitates were then electrophoresed and immunoblotted with anti-PSGL-1 mAb; **(D)** CD44 immunoprecipitates (CD44 IP) from equivalent amounts of cell lysates of CD14-S and PA-S mo-DCs were immunoblotted with E-Ig chimera or anti-CD44 mAb. **(E)** *Blot rolling assay of CD44 immunoprecipated from CD14-S mo-DCs.* CD44 immunoprecipitates were resolved by SDS-PAGE, blotted and stained with anti-CD44 mAb (blot below the graph). E-selectin-transfected CHO cells (CHO-E)

were perfused over blots at 1.4 dyn/cm^2 and then shear stress was increased to 4.2 dynes/cm^2. E-selectin-dependent tethering and rolling was observed at the CD44 band (arrow). Assays were performed in the presence or absence of 5 mM EDTA, or following preincubation of CHO-E with isotype control mAb or function blocking anti-human E-selectin mAb (clone 68-5H11).

Ligation of CD44 triggers β1-integrin activation in CD14-S mo-DCs

To assess the relative contribution(s) of HCELL to the augmented E-selectin-mediated TEM of CD14-S mo-DCs, we compared the effects of engagement of CD44/HCELL and PSGL-1/CLA on VLA-4 function. We examined whether the ligation of each of the pertinent scaffold proteins, CD44 and PSGL-1, to their canonical ligands, hyaluronic acid (HA) and P-selectin, respectively, alter VLA-4 binding activity. Notably, exposure of CD14-S mo-DCs to HA (Supplemental Figure S2) consistently resulted in β1-integrin activation (Figure 3A), but exposure of PA-S mo-DCs to HA did not yield β1-integrin activation, nor did engagement of either CD14-S or PA-S mo-DCs with P-selectin (Figure 3A). Moreover, in CD14-S mo-DCs, but not PA-S mo-DCs, CD44 engagement with HA increased mo-DC adhesiveness to CS-1 fibronectin in a VLA-4-dependent manner (Figure 3B). These data highlight the predominant role of CD44 ligation in VLA-4 activation, thereby indicating that observed effects of E-selectin engagement on VLA-4 activation of CD14-S mo-DCs are mediated through HCELL.

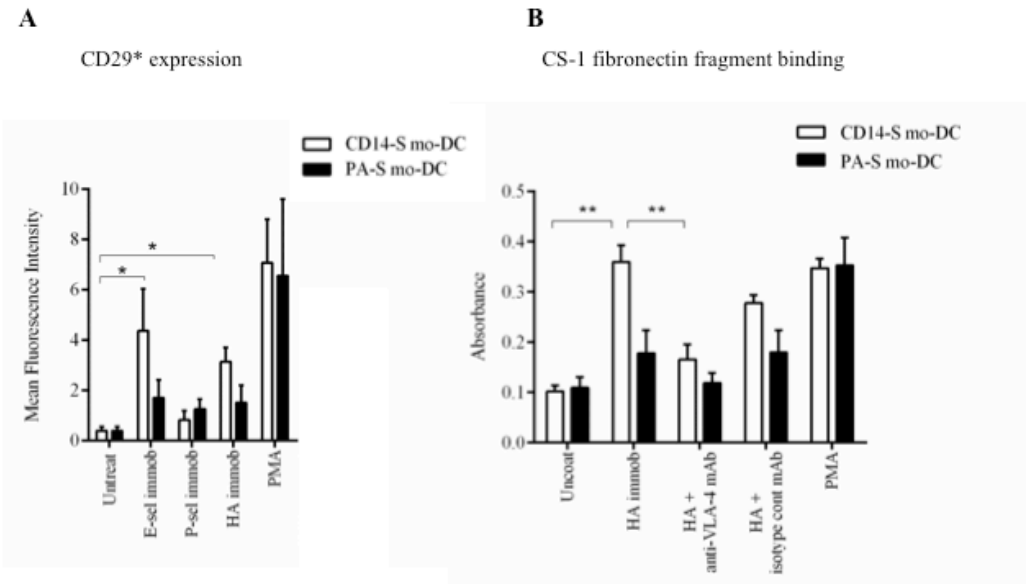

Figure 3. VLA-4 binding activity is triggered by engagement of CD44 on CD14-S mo-DCs. (A) *Flow cytometry analysis of activated β1-integrin expression.* Expression of the activation-dependent epitope of CD29 (HUTS-21) was assessed on CD14-S mo-DCs (white bars) and PA-S mo-DCs (black bars) incubated on plates coated with either E-selectin, P-selectin, or HA, or stimulated with PMA. Graph values represent the mean fluorescence intensity of HUTS-21 mAb staining (CD29*) of mo-DCs (n= 3, mean ± SD). **(B)** *Adhesion of*

CD14-S and PA-S mo-DCs to CS-1 fibronectin fragment. Mo-DCs were incubated on uncoated plates or plates coated with HA, and then collected for analysis of binding to CS-1 peptide coated on plates. Integrin activation by treatment with PMA was used as positive control; the numbers of CS-1-adherent cells were quantified by light absorbance (595 nm) following crystal violet staining. As shown in the Figure, binding to HA by CD14-S mo-DCs, but not by PA-S mo-DCs, induced VLA-4 adhesion to CS-1 peptide, which was abrogated by treatment with anti-VLA-4 blocking mAb (HP2/1). Values are means ± SD (n= 3). Statistically significant differences ($P \leq 0.05$) related to HA engagement are indicated by brackets and asterisks.

TNFα-treatment boosts TEM of CD14-S mo-DCs

The inflammatory cytokine TNFα induces DC maturation[30] and is routinely used in clinical applications.[31] We thus assessed whether TNFα treatment could affect mo-DC TEM ability. When treated with TNFα, both CD14-S and PA-S mo-DCs showed characteristic increased expression of MHC Class II antigens and the co-stimulatory moleculeCD86,[32] however, only CD14-S mo-DCs displayed increased transmigration on stimulated HUVEC (Figure 4A). TEM of CD14-S mo-DCs was abrogated by function-blocking anti-E-selectin mAb, by sialidase treatment of mo-DCs, by function-blocking anti-VLA-4 mAb and by PTX treatment of mo-DCs, confirming a role for both E-selectin receptor/ligand interactions and G-protein-signaling in driving VLA-4-dependent adhesive interactions (Figure 4A).

To determine whether the enhanced CD14-S mo-DC TEM following TNFα treatment could be secondary to changes in integrin levels, we measured the cell surface expression of the heterodimer chains of VLA-4 and LFA-1. While no increase was observed on the cell surface expression of the CD29, CD18 or CD11a integrin subunits, surface expression of CD49d was considerably augmented after TNFα treatment (Figure 4B). TNFα-treated mo-DCs also showed a significantly increased expression of the activation epitope of β1-integrin upon engagement on CHO-E cells and, commensurately, displayed increased VLA-4 binding to the CS-1 fibronectin fragment (Figure 4C). Altogether, these data indicate that TNFα induces VLA-4 expression on CD14-S mo-DCs, and confirms a role for E-selectin receptor/ligand interactions in priming VLA-4 integrin activation in these cells.

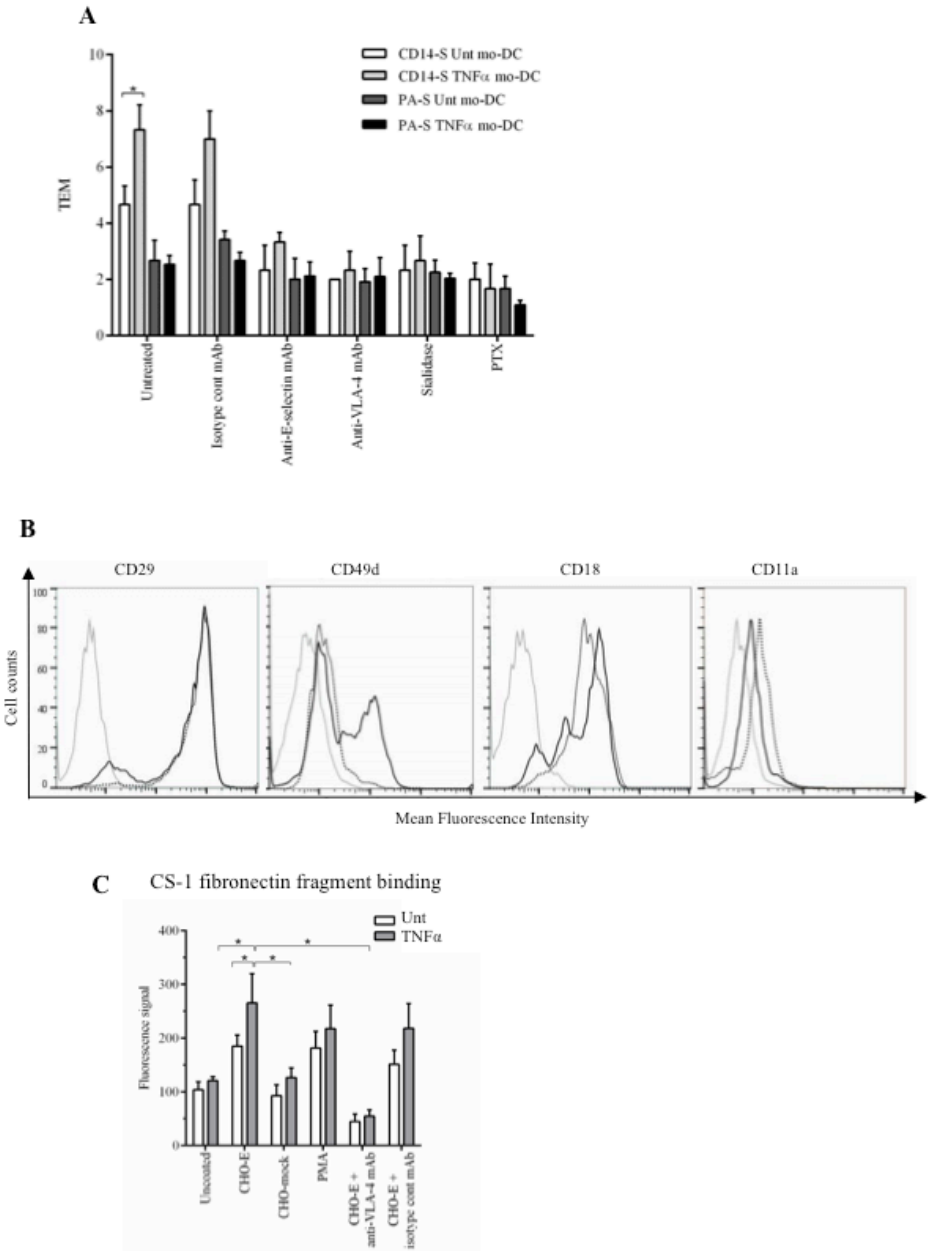

Figure 4. TNFα-treatment of mo-DCs affects TEM and VLA-4 activity. (A) *Transendothelial migration (TEM) assay of untreated (Unt) and TNFα-treated CD14-S and PA-S mo-DCs*. The relative TEM value was calculated as the ratio of transmigrated cells on TNFα-stimulated HUVECs compared with cells transmigrated on non-stimulated HUVECs. TEM values were analyzed for HUVECs preincubated with function blocking anti-E-selectin mAb clone 68-5H11, for mo-DCs preincubated with function blocking anti-VLA-4 mAb HP2/1 or isotype mAb, and for mo-DCs treated with sialidase or PTX. **(B)** *Flow cytometry analysis of cell surface expression of integrins VLA-4 and LFA-1 on CD14-S mo-DCs*. Histograms show the staining in untreated (dotted black line) and TNFα-treated (solid black line) mo-DCs. Grey lines represent isotype control. **(C)** *Analysis of CD14-S mo-DCs binding to CS-1 fibronectin fragment*. Adhesion of untreated (Unt) or TNFα-treated CD14-S mo-DCs (TNFα) to CS-1 peptide was assessed following incubation of mo-DCs on plates containing monolayers of E-selectin-transfected CHO cells (CHO-E), mock transfected CHO (CHO-mock), or plates containing no CHO cells (uncoated). After incubation, cells were collected for binding to CS-1 peptide coated on plates. The number of CS-1-adherent cells was quantified by light absorbance (595 nm) following crystal violet staining. Cells activated with PMA (positive control) bound avidly to CS-1, and pre-incubation of cells on CHO-E markedly augmented binding to CS-1. Incubation with anti-VLA-4 blocking antibody (HP2/1) for the last 15 min of CHO-E engagement (CHO-E+anti-VLA-4 mAb) abrogated binding to CS-1. Values are mean

± SD (minimum of n=4). Statistically significant differences ($P \leq 0.05$) related to CHO-E engagement are indicated by brackets and asterisks.

TNFα treatment of CD14-S mo-DCs sustains E-selectin ligand expression during culture

To assess whether the differences in TEM and integrin activation in CD14-S mo-DCs cultured in presence or absence of TNFα reflected differences in expression of E-selectin ligands, we performed flow cytometry, parallel plate based assays, and western blot analysis to assess E-selectin binding of these cells. Following differentiation of monocytes into mo-DCs, there was no difference in flow cytometry profile of E-Ig staining between day 0 CD14-S cells (Figure 2A) and those cells cultured for three days in absence ("untreated") or presence of TNFα ("matured mo-DCs") (Figures 5A). However, compared to untreated CD14-S mo-DCs, TNFα-treated CD14-S mo-DCs showed markedly lower rolling velocity on stimulated HUVECs under hemodynamic fluid shear conditions (indicative of increased E-selectin avidity) (Figure 5B), and western blot analysis showed significantly greater E-Ig staining of lysates (Figure 5C). Specifically, compared with cells at start of culture (Day 0 cells), Day 3-cultured untreated mo-DCs showed significantly reduced levels of CLA and HCELL (i.e., E-Ig reactive bands at~120-130 kDa and ~80-90 kDa, respectively), whereas TNFα-treated mo-DCs showed sustained E-Ig reactivity, with blot profile similar to that observed on Day 0 (Figure 5C). In contrast to CD14-S mo-DCs, the blot profile of E-Ig reactivity of PA-S mo-DCs was unchanged by TNFα treatment (Supplemental Figure S3). Notably, though E-Ig staining patterns by western blot were markedly different, CD44 levels were only slighter higher on CD14-S mo-DCs between Day 0 and Day 3 of culture (Figure 5D), indicating that observed markedly increased HCELL expression (i.e., higher E-Ig reactivity) reflects increased content of sLe^x on the CD44 scaffold and not changes in expression of the CD44 protein itself.

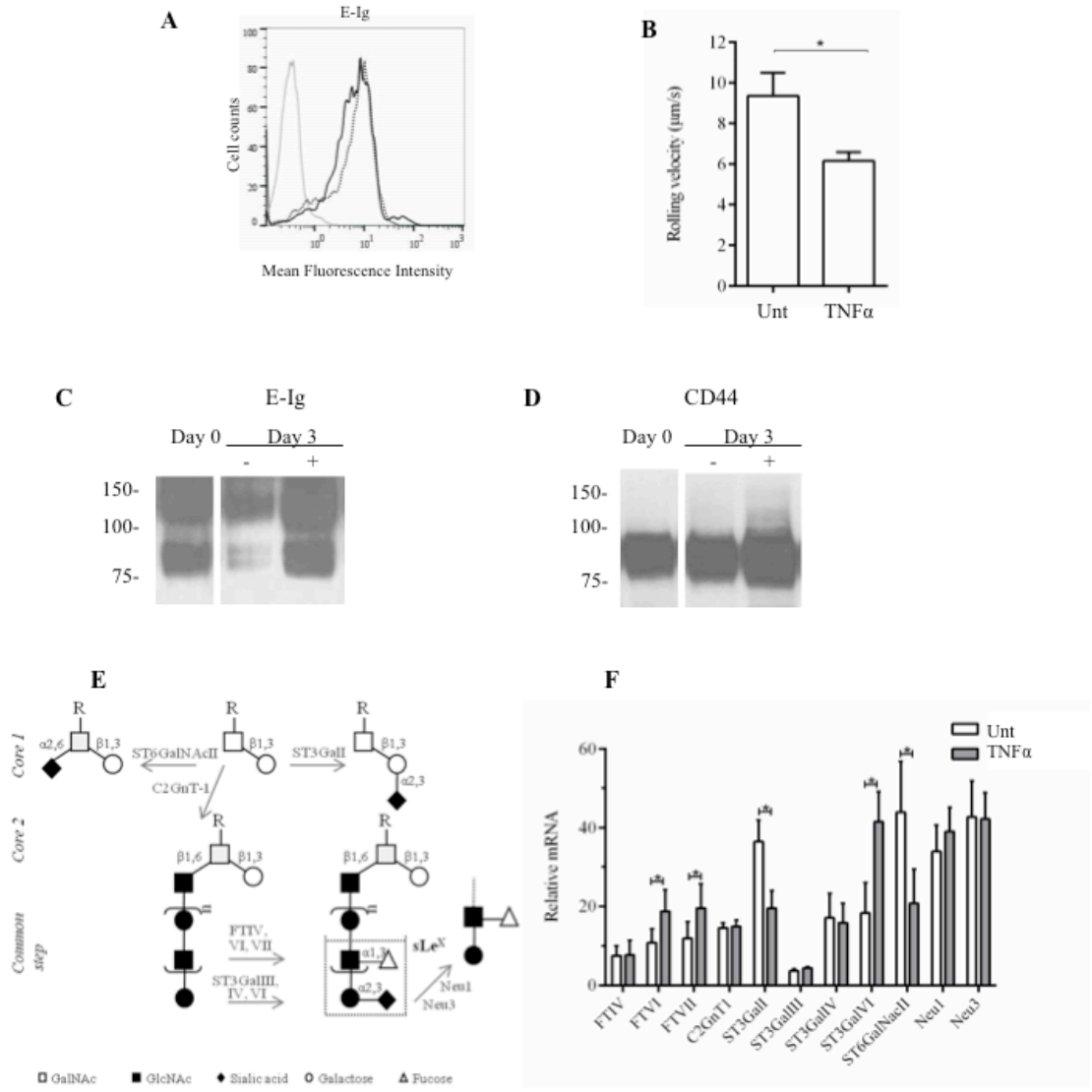

Figure 5. E-selectin ligand expression on cultured human CD14-S mo-DCs is preserved by TNFα treatment. (A) *Flow cytometry analysis of E-Ig reactivity on CD14-S mo-DCs*. Representative histograms of E-Ig staining of untreated mo-DCs (dotted black line) and mo-DCs treated with TNFα (solid black line) collected after 3 days of culture. Grey line represents E-Ig staining in absence of Ca^{2+}. **(B)** *Rolling adhesive interactions on TNFα-stimulated HUVECs of cultured CD14-S mo-DCs untreated (Unt) or treated with TNFα (TNFα) for 3 days*. Rolling velocities were measured at shear stress of 2.0dyn/cm^2. The velocity was calculated by measuring the displacement of the centroid of the cell over 1 sec.; lower rolling velocity of TNFα moDCs indicates higher avidity to E-selectin. **(C)** *Western blot analysis of E-Ig reactivity of whole cell lysates of CD14-S mo-DCs*. Cells were treated (+) or not (-) (at day 0) with TNFα and collected at day 3 after treatment. TNFα-treated mo-DCs show higher E-Ig reactivity at bands at ~130 kD and ~80 kD. **(D)** *Western blot analysis of CD44 expression of CD14-S mo-DCs*. Cells were collected at day 0 and day 3 with (+) or without (-) TNFα treatment. As shown in the figure, TNFα treatment sustains the expression of HCELL in vitro. **(E)** *Schematic of sLe^X biosynthesis on N- and O-glycans.* Enzymes that were analyzed in this study are indicated in grey next to the arrows: core 2-synthase (C2GnT1), sialyltransferases (ST3GalI, III, IV and VI, ST6GalNAcII), fucosyltransferases (FTIV, VI and VII), and sialidases (Neu1, Neu3). **(F)** *Real-time PCR analysis showing relative expression of selected genes.* The relative mRNA level was computed as the permillage fraction (‰, i.e., the proportion of target mRNA per thousand of the reference *GAPDH* mRNA expression) of mo-DCs treated for 3 days with TNFα (grey bars) or left untreated (white bars). Values are means ± SD (n= 3). Statistical significant differences ($P \leq 0.05$) are indicated by asterisks.

The E-selectin binding determinant sLe^x is displayed on *O*-glycans in CLA[33] and on *N*-glycans in HCELL.[34] To assess whether TNFα induces expression of enzymes critical to the biosynthesis of sLe^x[11,13,35] (Figure 5E), we performed RT-PCR to analyze expression of the α-(1,3)-fucosyltransferases FTIII, FTIV, FTVI and FTVII, and the α-(2,3)-sialyltransferases ST3GalIII, ST3GalIV, and ST3GalVI, which add fucose and sialic acid, respectively, to lactosaminyl glycan precursors of sLe^x.[36] Moreover, we evaluated the expression of the core 2-synthase, C2GnT1, which forms the scaffold glycan for polylactosamine extension on *O*-glycans, and the enzymes ST3GalI and ST6GalNAcII, each which act on core 1 *O*-glycans and antagonize core 2 extensions (Figure 5E), thereby down-regulating *O*-glycan expression of sLe^x.[11,37] We also analyzed the expression of Neu1 and Neu3 sialidases in mo-DCs,[38] which are known to remove sialic acid moieties from sLe^x.[35] As shown in Figure 5F, TNFα-treated mo-DCs showed significantly more expression of FTVI, FTVII and ST3GalVI and less expression of ST3GalI and ST6GalNAcII, compared to that of untreated cells, with no differences in RNA transcripts encoding sialidases Neu1 and Neu3. Altogether, these changes in glycosyltranserase gene expression would serve to augment display of cell surface sLe^x on both *O*- and *N*-glycans.

FTVI treatment increases the expression of functional E-selectin ligands and increases E-selectin ligand/VLA-4-dependent transendothelial migration of CD14-S mo-DCs.

HCELL is prominently expressed by human hematopoietic stem/progenitor cells, including the human hematopoietic progenitor cell line KG1a.[39] Compared with KG1a cells, the levels of E-selectin ligands and of HCELL expressed by CD14-S mo-DCs are relatively modest (Figure 6A). We thus sought to determine whether stereoselective α-(1,3)-exofucosylation with FTVI[27] could boost surface expression of E-selectin ligands. Following FTVI treatment of mo-DCs, we consistently observed a considerable increase in E-Ig reactivity in both CLA and HCELL (Figure 6B), and exofucosylated CD14-S mo-DCs displayed increased transmigration (~2-fold more) compared to buffer-treated mo-DCs (Figure 6C). Notably, exofucosylation of PA-S mo-DCs also increased E-Ig binding, with increased reactivity at ~130 kDa (consistent with CLA) and *de novo* expression of a faint E-Ig-reactive ~80-90 kDa band (consistent with HCELL; Supplemental Figure S4). However, PA-S mo-DCs treated with FTVI displayed no significant change in transmigration compared to buffer treated mo-DCs (Supplemental Figure S4).

To further analyze the effect of FTVI treatment on CD14-S mo-DCs, we assessed CD14-S mo-DC binding to unstimulated and TNFα-stimulated HUVECs under physiological shear stress conditions. On stimulated HUVECs, the mo-DCs exhibited a modest capacity to tether and roll, with cells transitioning to firm adherence under flow (Figure 6D). Notably, FTVI treatment of CD14-S mo-DCs led to a significant increase in the number of tethering/rolling and firmly adherent cells and markedly decreased rolling velocity compared to that of buffer treated (BT) cells (Figures 6D and 6E), indicating increased adhesion to E-selectin. FTVI treatment did not alter the expression levels of CD44 or PSGL-1 or of other proteins involved in cell trafficking such as β1- and β2-integrins (Supplemental Figure S6), suggesting that the decreased rolling velocity of α-(1,3)-exofucosylated mo-DCs was primarily due to increased levels of cell surface E-selectin ligands.

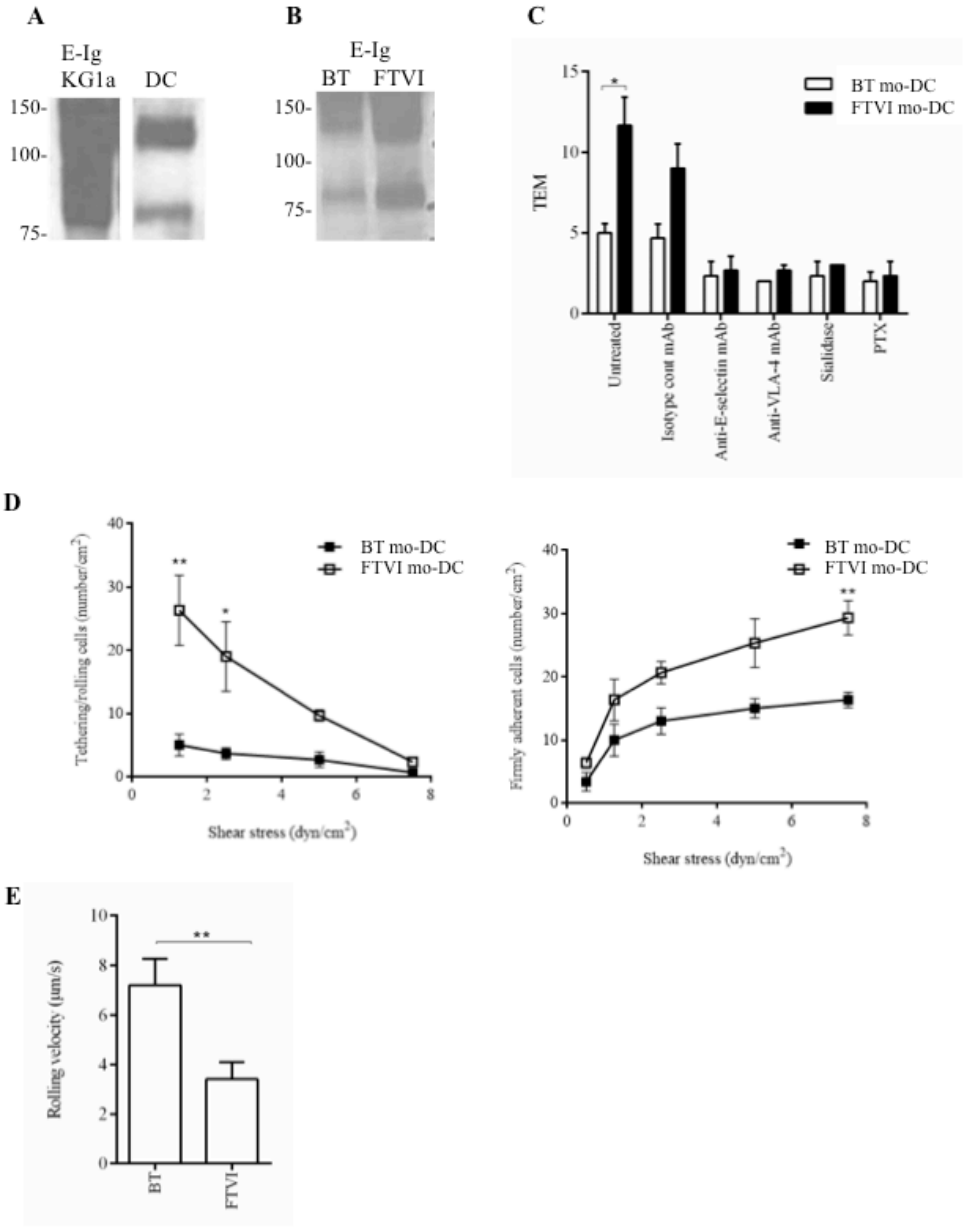

Figure 6. Enforced fucosylation of human CD14-S mo-DCs yields higher E-selectin binding. (A) *Western blot analysis comparing E-selectin binding (E-Ig reactivity) of KG1a and CD14-S mo-DCs*. Input lysates on each blot were normalized to equivalent cell numbers. **(B)** *Effects of exofucosylation on E-selectin ligand expression of mo-DCs*. CD14-S mo-DCs were buffer treated (BT) or FTVI-treated, and lysates (of equivalent cell numbers) were analyzed by Western blot for reactivity with E-Ig. (C) *Transendothelial migration (TEM) assay of buffer treated (BT) and FTVI-treated mo-DCs*. The relative TEM value is the ratio of mo-DCs transmigrated on TNFα-stimulated HUVECs compared with mo-DCs transmigrated on non-stimulated HUVECs. TEM was assessed using mo-DCs (untreated), HUVECs preincubated with function blocking anti-E-selectin mAb clone 68-5H11, mo-DCs preincubated with function blocking anti-VLA-4 mAb HP2/1 or isotype mAb, and mo-DCs treated with sialidase or PTX. (D) *Analysis of mo-DCs tethering/rolling interactions and firm adherence on TNFα-stimulated HUVEC monolayers under flow conditions. Left:* Shear stress was increased stepwise every 30 seconds, and the number of tethering/rolling cells per cm^2 of HUVEC was counted, during each 30 second segment. *Right*: At the end of each time segment, the number of cells that became firmly adhering per cm^2 area was measured. (E) *Average rolling velocity of BT- and FTVI-treated mo-DCs perfused over TNFα-stimulated HUVECs*. The velocity was estimated at 2.0 dyn/cm^2 flow, as the displacement of the centroid of the cell over 1 sec. Values are means ± SD (n= 3). Statistically significant differences are indicated by asterisks.

Discussion

There is great hope that DC-based immunotherapy approaches will yield improved outcomes for a variety of conditions including cancer, infectious diseases and autoimmune diseases. A proximate hurdle in achieving this promise is to deliver the cells to relevant tissue(s) where they can then encounter cognate antigen(s) for presentation to immunologic effectors. Human DCs can be generated in vitro from human monocyte precursors (mo-DCs)[30] in sufficient numbers for clinical applications following CD14 immunomagnetic separation (CD14-S) and plastic adherence separation (PA-S) of blood monocytes.[40] It has been reported that these different monocyte isolation methods may affect phenotypic proprieties of the generated DCs[5,41], yet it is unknown whether different monocyte enrichment techniques could have subsequent effects on DC-endothelial interactions critical to extravasation at target tissues. Accordingly, in this study, we examined DCs derived from both PA and CD14-S monocyte selection for their capability to undergo transendothelial migration (TEM), and the adhesion molecules mediating TEM for each of these mo-DC populations.

To compare E-selectin ligand expression in each mo-DC population, we performed flow cytometry and western blot analysis using E-Ig as a probe. By flow cytometry, E-Ig reactivity was slightly higher in CD14-S mo-DCs compared to PA-S mo-DC, but Western blot revealed a striking difference in the profile of E-selectin ligands: while both mo-DC types showed robust staining at a ~120-130 kDa band representing the monomer form of CLA (the glycovariant of PSGL-1 that binds E-selectin), only CD14-S mo-DCs showed an E-Ig-reactive glycoprotein at a ~80-90 kDa band. Biochemical studies indicated that this second band represents HCELL, the specialized glycovariant of CD44 that binds E-selectin. Notably, the finding that HCELL is expressed on CD14-S mo-DCs is novel, as HCELL display was previously thought to be restricted only to primitive human hematopoietic progenitor cells and certain adenocarcinoma cells.[15,34,42]

Our studies reveal that engagement of CD14-S mo-DCs with E-selectin leads to a remarkable increase in VLA-4-mediated adhesiveness to TNFα-stimulated HUVEC monolayers. To examine the relative roles of HCELL and CLA in endothelial adhesive interactions, mo-DCs were exposed to hyaluronic acid and to P-selectin, ligands specific to the protein scaffolds CD44 and PSGL-1, respectively. Importantly, we observed that ligation of CD44, but not PSGL-1, induced VLA-4 activation, indicating that E-selectin ligation of HCELL, and not CLA, drives VLA-4-mediated adhesiveness of mo-DCs.

In contrast to the canonical multistep model of cell migration, whereby engagement of chemokine receptor(s) (step 2) is obligatory to achieve integrin activation (step 3), we observed that HCELL ligation by E-selectin alone triggers PTX-inhibitable endothelial transmigration, consistent with a G-protein-dependent inside-out stimulation of VLA-4 activity. This capacity of E-selectin to initiate mo-DC transmigration is due to the fact that sLe^x binding determinants are expressed on the CD44 scaffold, thereby engendering CD44 ligation. Though this CD44-dependent "step 2-chemokine bypass pathway" has been described in human mesenchymal stem cells following enforced HCELL expression by cell surface glycan engineering,[26,27] these results are the first to indicate that natively-expressed HCELL and VLA-4 can function in a bimolecular axis to drive transmigration of a human leukocyte.

TNFα is widely used to promote the maturation of DCs in immunotherapy.[31] This pro-inflammatory cytokine characteristically increases the expression of E-selectin and VCAM-1 on endothelial cells,[10,43] but the effect of this cytokine on E-selectin ligands expression or on VLA-4 expression on mo-DCs was previously unknown. Our data show that TNFα preserves the expression of HCELL on culture-expanded CD14-S mo-DCs, which otherwise undergoes time-dependent decay following differentiation of DC from monocytes. TNFα also increases expression of VLA-4, and, therefore, the combined effect on HCELL and VLA-4 expression would license augmented β1-integrin activation and consequent TEM on endothelial beds expressing both E-selectin and VCAM-1.

In agreement with previous reports,[13,44] our data indicate that TNFα induces significant changes on glycosyltransferase gene expression in CD14-S mo-DCs. As compared to untreated CD14-S mo-DCs, TNFα-treated DCs express lower levels of transcripts encoding ST3GalI and ST6GalNAcII, and higher levels of that of C2GnT1. This pattern would promote the expression of core 2 glycan structures that are requisite for elaboration of the CLA glycoform of PSGL-1. In addition, TNFα treatment upregulates expression of the fucosyltransferases FTVI and FTVII and of the sialyltransferase ST3GalVI, which, collectively, heighten requisite α-(1,3)-fucosylation and α-(2,3)-sialylation of lactosamine chains to create sLe^x.[45] This pattern of glycosyltransferase gene expression would serve to sustain HCELL expression, as observed in TNFα–treated CD14-S mo-DCs.

Apart from cytokine effects on expression of Golgi glycosyltransferases, cell surface sLe^x expression can be modulatedvia stereospecific exoglycosylation.[26,46] In this study, we

explored whether α-(1,3)-exofucosylation with FTVI would program increased expression of mo-DC E-selectin ligands. FTVI places fucose in α-(1,3)-linkage to an acceptor N-acetylglucosamine located specifically within a terminal α-(2,3)-sialylated type 2 lactosamine unit (NeuAcα2-3Galβ1-4GlcNAcβ1-R, Figure 5E). We found that FTVI treatment of CD14-S mo-DCs increased both HCELL and CLA expression and markedly enhanced TEM ability. However, in contrast, FTVI treatment of PA-S mo-DCs did not augment HCELL expression, and, despite increased CLA expression, TEM was unchanged after FTVI treatment of PA-S mo-DCs. These findings indicate that glycoengineering to increase HCELL expression augments TEM of mo-DCs, whereas enforced CLA expression does not, therefore highlighting a key role for HCELL in licensing mo-DC TEM.

The results of our studies open new insights into the role of CD44 in DC biology. Our findings that human DCs express HCELL have important implications for elucidating the molecular basis of DC migration and for optimizing DC migration in adoptive cell therapeutics. The observation that HCELL is expressed by CD14-S mo-DCs but not by PA-S mo-DCs indicates that in vitro monocyte isolation/DC generation methods impact expression of E-selectin ligands and mo-DC transmigration. While TNFα is well-known to upregulate expression of E-selectin and VCAM-1 on endothelial cells, the data here indicate that TNFα also preserves the expression of E-selectin ligands on culture-expanded mo-DCs, thereby priming E-selectin- and VLA-4-dependent transmigration of mo-DCs (see model represented in Figure S6). Our findings thus offer a unifying perspective on the role of TNFα in recruitment of DCs to sites of inflammation. Altogether, the results of this study yield novel insights into functional diversity of CD44 in the biology of DC TEM, and also raise the possibility that expressly modifying HCELL expression in mo-DCs could improve the clinical efficacy of DC-based therapeutic strategies.

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Authorship Contributions and Disclosure of Conflicts of Interest

The study was conceived by both R.S. and P.V. R.S. designed and supervised all research, funded the research and wrote the manuscript. P.V. designed and performed research, collected and analyzed data, and wrote the manuscript. M.S. performed research and collected and analyzed data, and wrote the manuscript. According to the NIH policies and procedures, the Brigham & Women's Hospital has assigned intellectual property rights regarding HCELL to the inventor (R.S.), who may benefit financially if the technology is licensed. R.S.'s ownership interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policy.

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Supplemental Data

Supplemental Methods

Antibody reagents and flow cytometry

Cell surface staining for flow cytometry was performed using commercially-available mouse E-selectin-human Fc Ig chimera (E-Ig) and antibody reagents. Monocyte purity was evaluated by flow cytometry for expression of CD14, and differentiation and maturation of mo-DCs were evaluated by staining for expression of MHC-II, and CD86 (mAbs from BD Biosciences). Anti-CD44 mAb 2C5 and recombinant mouse E-selectin-human Fc Ig chimera (E-Ig) were from R&D Systems. HECA-452, anti-CD29 and anti-CD49d mAbs were from BD Biosciences. Activation-dependent epitopes of VLA-4 and LFA-1 were evaluated using mAb HUTS-21 (specific for β1 chain (CD29) (BD Biosciences)) and mAb 24 (specific for integrin β2 (CD18) chain of CD11/CD18 heterodimers (Hycult Biotech)), respectively.

Blot Rolling Assay

Western blots of PVDF membrane preparations stained with anti-CD44 were rendered transparent with Hanks balanced salt solution (HBSS) containing 2 mM Ca^{2+} and 10% glycerol and were placed in the parallel plate flow chamber. Chinese hamster ovary (CHO) cells transfected with full-length human E-selectin cDNA (CHO-E) were suspended (10^6 cells/mL) in HBSS containing 2 mM Ca^{2+} and perfused over blots at 1.4 dyn/cm^2 and incremented up to 4.2 dyn/cm^2. The number of cells rolling within and outside relevant immunostained bands of the blot was recorded and quantified within each field of view by video microscopy. Controls for non-specific binding consisted of CHO-E cells in media containing 5 mM EDTA (chelating Ca^{2+} to prevent E-selectin adhesion), and incubation of CHO-E cells in presence of function-blocking anti-E-selectin mAb. Mock transfectants (CHO-mock) were used as control to analyze background levels of CHO adhesive interactions on blots.

CS-1 fragment binding assay following E-selectin and hyaluronic acid engagement

Mo-DCs were placed in culture plates seeded with CHO-E or with mock transfectants (CHO-mock), or coated with E-Ig (10 μg/mL) or high molecular weight hyaluronic acid (HA, Sigma) (5 μg/μL). Mo-DCs, suspended in HBSS containing 2 mM Ca^{2+}, were placed onto the various plates for 30 min on a rocker platform (5-10 rpm). The cells were collected by vigorous pipetting, washed in HBSS without Ca/Mg, and then placed onto tissue culture plates previously coated with connecting segment-1 peptide (CS1) (Fibronectin-40 (Millipore, 10 μg/mL)). Mo-DCs were allowed to bind for 45 min at 37ºC in RPMI-1640/10 mM HEPES/0.2% BSA. Plates were then washed and adherent cells were identified by either crystal violet staining (absorbance 595 nm) or CFSE labeling (fluorescence signal).

Immunoprecipitation

Cell lysates were precleared with protein G-agarose (Invitrogen) and then incubated with anti-CD44 mAb (2C5) or anti-PSGL-1 mAb (KPL-1). For immunoprecipitation using E-Ig chimera, lysis buffer contained 2mM Ca^{2+}. Antibody or E-Ig immunoprecipitates were collected using protein G-agarose beads, which were washed with lysis buffer, pelleted and then resuspended in reducing sample buffer. The bead suspension was boiled, and supernatant (released protein) was subjected to SDS-PAGE, transferred to PVDF membrane, and blots were immunostained.

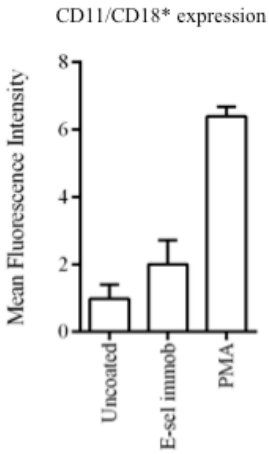
Fucosyltransferase VI treatment

Cells were treated with 60 mU/mL FTVI (Sigma), in HBSS containing 20 mM HEPES, 0.1% human serum albumin (HSA, Sigma) and 1 mM GDP-fucose (CarboSynth) for 45 min at 37ºC. Control consisted of cells treated with buffer containing all components except FTVI enzyme. The efficacy of the FTVI treatment was confirmed by evaluating for increased expression of E-selectin ligands as assessed by both flow cytometry and western blot analysis using E-Ig chimera as probe.

Supplemental Figures

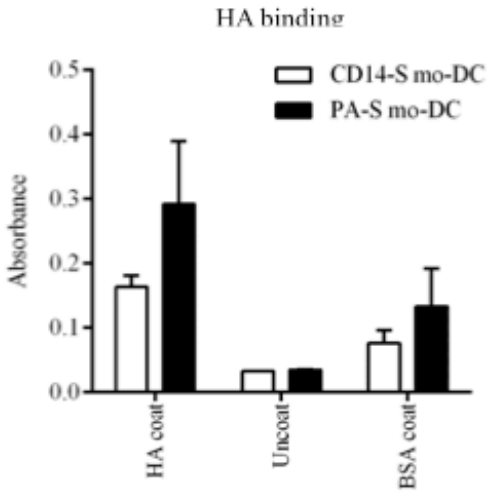
Table S1. Pairs of primers used in Real Time PCR assays.

Protein	Forward Primer (5'–3')	Reverse Primer (5'–3')
FTIV	GATCTGCGCGTGTTGGACTA	GAGGGCGACTCGAAGTTCAT
FTVI	GCGTGTGTCTCAAGACGATCC	GGAAGCGGGACCCATTAGG
FTVII	CACCTGAGTGCCAACCGAA	CACCCAGTTGAAGATGCCTCG
C2GnT1	AACCCCTTAGTAAAGAAGAGGCG	AGCAGCCTGTCAAGCATTTCA
ST3GalI	AAGAGGACCCTGAAAGTGCTC	CTCCAGGACCATCTGCTTGG
ST3GalIII	GCCTGCTGAATTAGCCACCAA	GCCCACTTGCGAAAGGAGT
ST3GalIV	CTTCCTGCGGCTTGAGGATTA	CTCACTCCCCTTGGTCCCATA
ST3GalVI	ACTGCATTGCATATTATGGGGAA	TGGCTTTGATAAACAAGGCTGG
ST6GalNAcII	ACTTCCGTGGCCTGTTCAATC	GGCGATGACTTGGTGAGAGAG
Neu1	GGAGGCTGTAGGGTTTGGG	CACCAGACCGAAGTCGTTCT
Neu3	AAGTGACAACATGCTCCTTCAA	TCTCCTCGTAGAACGCTTCTC



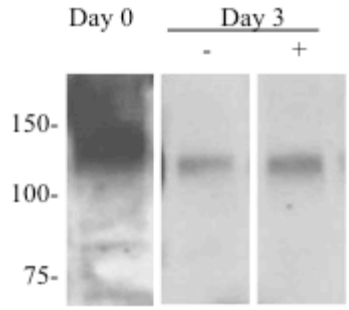
Supplemental Fig. S1

E-selectin ligand engagement on CD14-S mo-DCs does not lead to direct LFA-1 activation. Mo-DCs were incubated on uncoated or on E-selectin coated plates, or stimulated with PMA, and then stained with mAb 24 that recognizes an activation-dependent epitope of CD11/CD18 β2-integrin (CD11/CD18*). Graph values represent the mean fluorescence intensity of mAb 24 staining (n= 3, mean ± SD), subtracted from the values obtained using isotype control.



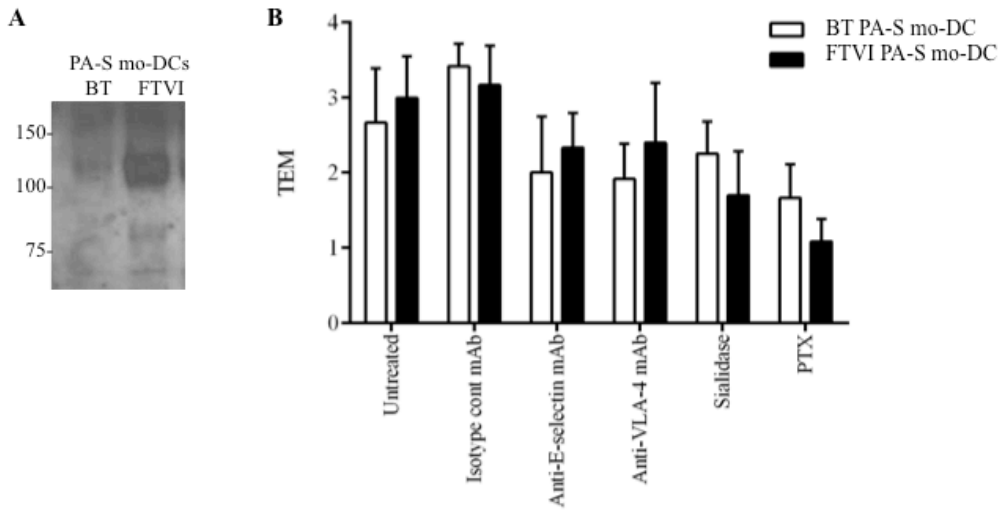
Supplemental Fig. S2

CD14-S and PA-S mo-DCs adhere to hyaluronic acid. CD14-S or PA-S mo-DCs were incubated on plates coated with hyaluronic acid (HA). The number of HA-adherent cells was quantified by light absorbance at 595 nm following crystal violet staining. Negative controls consisted on uncoated or BSA coated plates. Values are mean ± SD (n= 3).



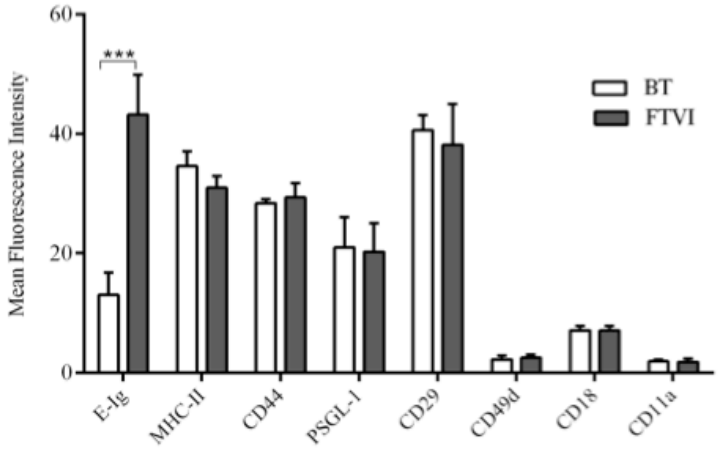
Supplemental Fig. S3

TNFα treatment on human PA-S mo-DCs does not preserve E-selectin ligand expression. PA-S mo-DCs were treated (+) or not (-) with TNFα and collected at day 3 after treatment. Lysates of equivalent cell numbers were analyzed by Western blot for reactivity with E-Ig. TNFα-treatment does not change E-Ig reactivity of PA-S mo-DCs.



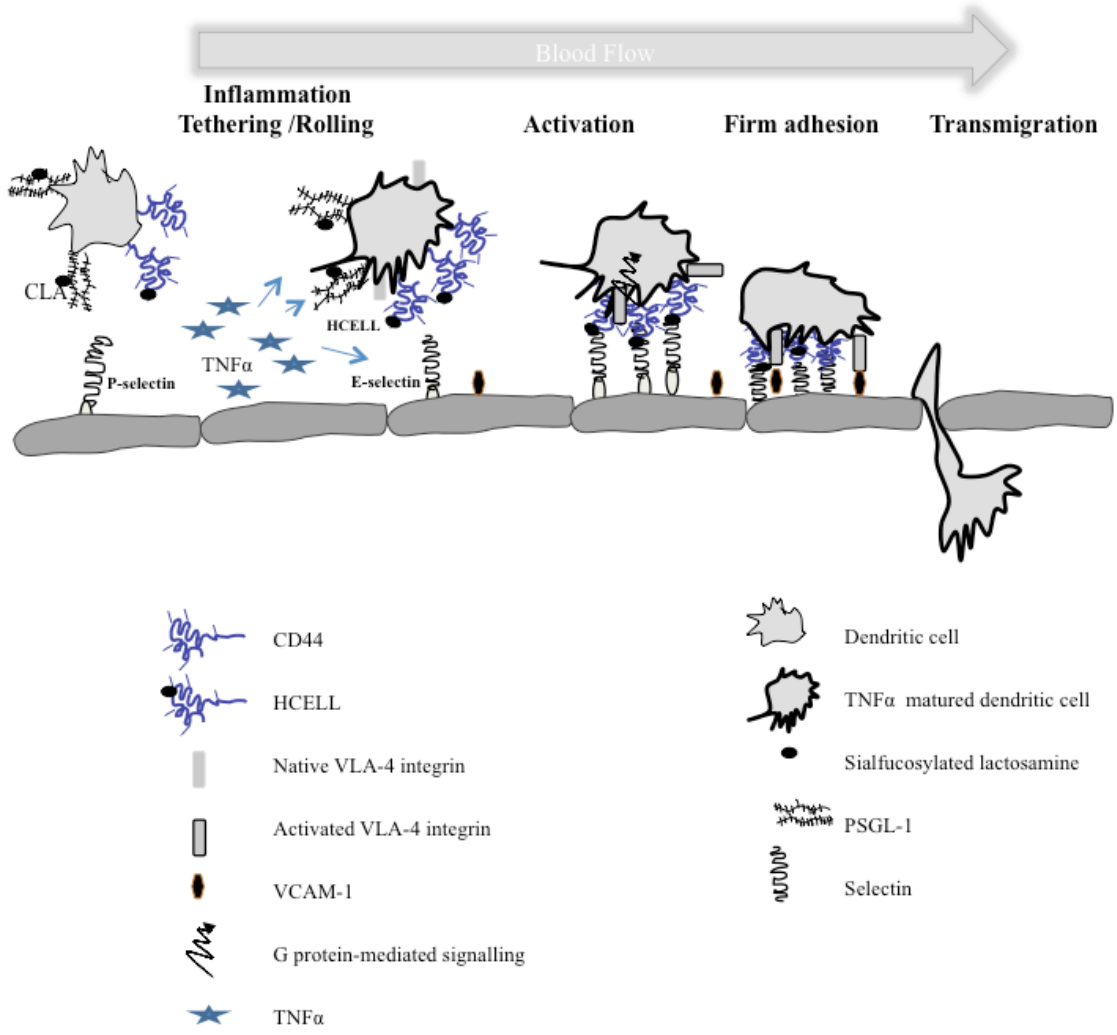
Supplemental Fig. S4

Enforced fucosylation of human PA-S mo-DCs increases E-Ig reactivity but does not affect transmigration. (A) *Western blot analysis of E-selectin binding (E-Ig) of buffer treated (BT) and FTVI-treated PA-S mo-DCs*. PA-S mo-DCs were buffer treated (BT) or FTVI-treated, and lysates (of equivalent cell numbers) were analyzed by Western blot for reactivity with E-Ig. (B) *Transendothelial migration (TEM) assay of buffer treated (BT, white bars) and FTVI-treated mo-DCs (grey bars).* The relative TEM value is the ratio of PA-S mo-DCs transmigrated on TNFα-stimulated HUVECs compared with PA-S mo-DCs transmigrated on non-stimulated HUVECs. TEM was assessed using mo-DCs (untreated), HUVECs preincubated with function blocking anti-E-selectin mAb clone 68-5H11, mo-DCs preincubated with function blocking anti-VLA-4 mAb HP2/1 and mo-DCs treated with sialidase or PTX. Values are mean ± SD (n= 3).



Supplemental Fig. S5

FTVI treatment does not affect cell surface protein expression. Flow cytometry analysis of E-Ig reactivity, MHC-II, CD44, PSGL-1, CD29, CD49d, CD18 and CD11a staining on buffer treated (BT, white bars) and FTVI-treated CD14-S mo-DCs (dark grey bars). Statistically significant differences are indicated by asterisks.



Supplemental Fig. S6

Schematic representation of molecular events that occur in the human dendritic cell (DC) extravasation process. In inflamed tissues, post-capillary endothelial cells express E-selectin and VCAM-1, which are induced by pro-inflammatory cytokines such as TNFα. TNFα can also augment expression of E-selectin ligands, including HCELL, and VLA-4 on DCs. Engagement of HCELL on DCs with endothelial E-selectin leads to G-protein-mediated signaling and activates VLA-4 integrin, resulting in firm adhesion of DCs to endothelial cells and subsequent transmigration.

Chapter 5

Characterization of E-selectin reactive glycoproteins expressed on human and mouse dendritic cells

Paper III- ***Manuscript in preparation***

TITLE

Characterization of E-selectin reactive glycoproteins expressed on human and mouse dendritic cells

RUNNING TITLE

Species-specific and sources-specifc diversity in E-selectin ligand expression by Dendritic Cells

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KEY POINTS

(1) CD43E, HCELL and CLA, the E-selectin reactive glycoform of CD43, CD44 and PSGL-1, respectively, are expressed on human blood monocyte-derived DCs

(2) Murine bone marrow derived-DCs do not express any glycoprotein with E-selectin reactivity

Abstract

Dendritic cells (DCs) are the most effective and versatile antigen-presenting cells, and therefore, they have been used as anti-cancer vaccines. However, the success of this therapy is limited by the fact that most of the adoptive transferred DCs do not reach the target tissue from the vasculature. Therefore, strategies to control the binding of DCs to endothelium are essential to improve their recruitment and to induce a more rapid and accurate antitumor immune responses. Thus, we analyzed the cognate E-selectin ligand(s) on human monocyte-derived DCs and murine DCs, obtained from different cellular sources. Westernblot analysis using E-selectin-Ig chimera (E-Ig) as a probe show that human mo-DCs generated from adult peripheral blood (APB) monocytes display great E-selectin binding. To define the relevant glycoproteins with E-selectin binding activity, lysates from human APB mo-DCs were immunoprecipitated using E-Ig and resolved by western blot. In addition to the already reported glycoforms of PSGL-1 "CLA" and of CD44 "HCELL", APB mo-DCs also express the functional E-selectin reactive glycoform of CD43 "CD43E". By contrast, umbilical cord blood (UCB) mo-DCs are devoid of E-selectin reactive glycoproteins, although they express lipid scaffolds that display E-selectin reactive determinants. Notably, enforced exofocusylation of both types of mo-DCs profoundly increased E-selectin adherence under hemodynamic shear stress conditions. Finally, murine DCs generated from bone marrow precursors (BM-DCs) or obtained from spleen (sDCs) show a completely different E-selectin ligand profile between them. Murine sDCs, but not BM-DCs, express CLA, whereas BM-DCs only display E-selectin reactive glycolipids. Our work describe the major glycoprotein E-selectin ligands of human and mouse DCs, disclosing critical species and cellular sources differences in E-selectin ligand expression among DCs.

Introduction

Dendritic cells (DCs) are unique in their ability to prime both *naïve* and memory T cell immune responses.[1] DCs arise from a common bone marrow myeloid hematopoietic progenitor, and after development, DC precursors egress the bone marrow and migrate via bloodstream to peripheral tissues, where they survey for signs of danger.[2] In the periphery, DCs capture antigens and following phenotypic changes migrate to secondary lymphoid organs, where they interact and activate T cells.[3] In addition, DCs are also able to activate *naïve* and memory B cells, NK cells and NKT cells and are one of the few cells capable of cross-presenting exogenous antigens to $CD8^{+}$T cells via Major Histocompatibility complex Class-I (MHC-I), a crucial mechanism for the generation of anti-tumor cytotoxic T cell responses.[3-5] Given these pivotal immune boost features, vaccination with autologous DCs pulsed with patient's tumor-antigens has been explored either in combination with classical chemotherapy or/and radiotherapy or after treatment, in order to prevent cancer relapses.[6-8] However, one of the immediate obstacles to anti-cancer DC-based vaccines success is the delivery of *ex vivo* generated DCs into target sites.[3] The tumor tissue is rich in infiltrating effector memory T cells.[9] Direct migration of *ex vivo* generated DCs to these sites would enhance local antigen-presentation to infiltrating T cells, and therefore, induce faster and stronger antitumor-specific cytotoxic T cell responses than priming directly *naïve* T cells in lymph nodes.[10,11] However, a recent study demonstrated that, in clinical trials involving these DC-based vaccines, a large number of injected DCs do not move from vascular to extravascular compartments and remained at the injection site.[12] Therefore, a greater understanding of the effectors mediating physiologic trafficking and tissue transmigration of vascularly-administered DCs is critical to accomplish the great benefit of DC-based anti-cancer vaccines, which rely on efficient delivery of these cells to target sites of disease in the patient.

To get into inflamed/tumor sites, *ex vivo* generated DCs have to engage through several adhesion contacts with endothelial cells via a cascade of molecular of events.[13] This process deeply depends on the initial adhesive interactions responsible by the slowing down of leukocyte rolling velocity along endothelium surface, to velocities well below the local blood flow rate. Vascular E-selectin and its counter ligands are the main mediators of leukocyte deceleration on target endothelium.[13] E-selectin is an inducible endothelial molecule expressed following inflammatory stimuli and, due to the inflammatory milieu, tumor

microvasculature displays an elevated concentration of E-selectin.[14,15] This molecule exhibits binding-affinity to glycoproteins and glycolipids decorated with sialyl Lewis X (sLe^X) – a terminal tetrassacharide modified with an α(2,3)- linked sialic acid substitution on galactose and an α(1,3) linked fucose modification on *N*-acetylglucosamine.[16]

Several glycoproteins have been identified and characterized as E-selectin ligands expressed on mature hematopoietic cells. These are the heavily sLe^X-decorated glycoforms of: PSGL-1 (known as Cutaneous Lymphocyte Antigen (CLA))[17], CD44 (known as Hematopoietic Cell E-/L-selectin ligand (HCELL)), CD43 (known as CD43E)[18], MGF-160 (known as E-selectin ligand-1 (ESL-1))[19], β2 integrins (CD18/CD11)[20] and L-selectin[21]. So far, only CLA[22] and HCELL (Videira et al., unpublished data) have been characterized as E-selectin ligands expressed on human DCs, whereas murine DCs transiently express CLA.[23] While CD43E is expressed by human and murine T cells[24,25] and by human monocytes (Silva et al., unpublished data), it is unknown whether it also plays a role as E-selectin ligand on DCs.

Accordingly, in the present study, we sought to perform a detailed analysis of the E-selectin glycoprotein ligands expressed in human and mouse DCs. For that, we used human monocyte derived-DCs (mo-DCs), obtained from two different blood sources (adult peripheral blood (APB) and umbilical cord blood (UCB)), and murine DCs, either derived from bone marrow (BM-DCs) or spleen (sDCs). Collectively, our findings reveal previously unsuspected differences in E-selectin ligand expression between different cellular sources of DCs, as well species-specific differences. Particularly notable is the demonstration of CD43E expression in human APB mo-DCs, in addition to HCELL and CLA, whereas UCB mo-DCs are generally devoid of E-selectin reactive glycoproteins. Our results also highlight the feasibility of exofucosylation as a platform technology for optimizing the trafficking properties of human mo-DCs, which are harnessed in adoptive immunotherapies.

Materials and Methods

Mice

Six to eight week old C57BL/6 mice were housed and bred at the animal facility of Instituto Gulbenkian Ciência (IGC, Oeiras, Portugal). Mice were sacrificed by carbon dioxide inhalation followed by cervical dislocation. The Institutional Animal Care and Use Committee (IACUC) approved all procedures, which were in agreement with the Federation of European Laboratory Animal Science Associations (FELASA) directives, approval ID number AO10/2010.

Generation of human mo-DCs

Human DCs were differentiated from monocytes, which were isolated from either adult peripheral blood (APB) or umbilical cord blood (UCB) from healthy donors, under protocols approved by the Institutional Review Board of Brigham & Women's Hospital (BWH), with informed consent provided as per the Declaration of Helsinki.

Briefly, after peripheral blood mononuclear cells (PBMCs) isolation by Ficoll-Paque density gradient centrifugation (Sigma-Aldrich, St Louis, MO), monocytes were enriched using anti-CD14 coated magnetic beads (Miltenyi Biotech, Auburn, CA). Monocytes were then cultured for 5 days at a concentration of 1×10^6 cells/mL in complete RPMI medium (RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100µg/mL penicillin/streptomycin and 1% pyruvate (all from Gibco, Grand Island, NY, USA)). For monocyte differentiation into DCs, 750 U/mL of interleukin-4 (IL-4, R&D, Minneapolis, MN, USA) and 1000 U/mL of recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D) were added to the culture every two days, as previously described.[26]

Generation of murine Bone marrow DCs

Tibias and femurs from C57BL/6J mice were removed and bone-marrow (BM) flushed out with RPMI medium. For lysis of erythrocytes, BM cells were ressuspended in a 0.17M Tris-HCl and 0.16M NH_4Cl solution for 5 min at room temperature. After washed, BM cells were

ressuspended in RPMI complete medium supplemented with 10 ng/mL of recombinant mouse GM-CSF (Immunotools, Friesoythe, Germany) and cultured at 1×10^6 cells/mL on six-well plates for 5 or 8 days.[27]

Isolation of CD11c$^+$ DCs from mouse spleen

Female C57BL/6J mice (6–8 weeks old) were subcutaneously injected with 2×10^6 of B16 melanoma cells secreting murine FMS-like tyrosine kinase 3 ligand (B16-Flt3-L), in order to induce a large production of splenic immature DCs (sDCs). After 15 days of tumor injection, mice were sacrificed, spleen collected and splenocytes obtained by mechanical disruption. Further CD11c$^+$ cells isolation was performed by positive immunomagnetic selection, using anti-CD11c magnetic Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany).

Flow cytometry

Monoclonal antibodies (mAbs) used include anti-CLA clone HECA-452, anti-human CD43 clone 1G10, anti-human PSGL-1/CD162 clone KPL-1, anti-human CD44 clone 515, anti-mouse PSGL-1 clone 4RA10, anti-mouse CD44 clone IM7, anti-mouse CD43 clone S7 (all from BD Biosciences), anti-human CD44 clone 2C5 (R&D Systems) and anti-human CD18 clone 7E4 (beckaman Coulter).

All antibody solutions were prepared at 10 μg/mL in PBS. Cells were incubated with primary antibody or isotype control for 30 min at 4ºC, washed twice and incubated with secondary antibody for 30 min at 4ºC. For E-Ig (R&D Systems, 10 μg/mL) staining, DPBS with Ca^{2+} and Mg^{2+} was used for all incubations, dilutions, and washing. The secondary antibody used was a biotinylated antihuman IgG (1:200) followed by streptavidin-PE (5 μg/mL) staining. Flow cytometry using Attune® Acoustic Focusing Cytometer (Life Techonologies, Grand Island, NY, USA) was performed and data collected were analyzed with FlowJo 10.0.6 (TreeStar).

Whole cell lysates preparation

Cell membrane proteins were isolated by sonication followed by incubation in lysis buffer containing 2% NP-40. In brief, cells were washed twice in PBS and resuspended in ice-cold lysis buffer containing 150mM NaCl, 50mM Tris-HCI (pH 7.4), 0.02% NaN_3, 20 µg/mL PMSF and protease inhibitor cocktail tablet (Roche). Cell suspension was subject to three rounds of sonication and to solubilize the membranes, NP-40 was added to a final concentration of 2% and the ruptured cells were left rotating overnight at 4ºC. After, lysates were centrifuged, and supernatants containing the proteins were collected. Amount of lysate in each lane was normalized to cell number for each western blots performed.

Western blot analysis

Protein samples were diluted 1:3 into 4X SDS/β-mercaptoethanol loading buffer (Boston products, Boston, USA), boiled and then resolved on 7.5% SDS-PAGE electrophoresis gels (Bio-rad Laboratories, Hercules, Ca). Subsequently, resolved proteins were transferred to polyvinylidene difluoride membranes (PVDF membranes, Bio-Rad) and blocked with 10% skimmed milk in TBS/0.1% Tween 20 for at least one hour. Western blots were probed with primary antibodies followed by incubation with appropriate HRP-conjugate antibodies and chemiluminescence detection using Lumi-Light Western blotting substrate (Roche).

Immunoprecipitation studies

To immunoprecipitate E-selectin binding proteins, cell lysate were prepared in an altered lysis buffer containing 2mM $CaCl_2$, otherwise cell lysates were prepared as mentioned above. Cell lysates were incubated with antibodies or E-Ig (each at 3µg of antibody per 6 million of cells) for 2 hours at 4ºC and then incubated with protein G-agarose for 4 hours at 4ºC under constant rotation. Antigen-antibody- bound protein G beads were washed then diluted in 1.5 X SDS/β-mercaptoethanol loading buffer and boiled to release antigens. The immunoprecipitates were subsequently subject to SDS-PAGE and western blotting.

Fluorescence microscopy

The expression of E-selection ligands was also analyzed by fluorescence microscopy. Monocytes from APB were allowed to adhere to poly-lisine coated cover glasses, and cultured for 6 days in the presence of GM-CSF and IL-4, as mentioned above. After blocking with 5% bovine serum albumin (BSA, Sigma) for 30 minutes, cells were stained with E-Ig for one hour, washed and incubated with appropriated fluorescent-conjugated secondary antibody. For F-actin staining, cells were permeabilized with triton and then incubated with phalloidin for 20 minutes. As a negative control, E-Ig binding was also tested in the presence of 20mM of EDTA. The cells were mounted on glass slides in 90% of glycerol solution. Images were acquired with a Leica TCS SP2 AOBS confocal microscope (Leica Microsystem GmbH, Mannheim, Germany) and assembled with Leica Confocal Software LCS Lite 2.6 (Leica Microsystem)

Blot rolling assay

The blot rolling assay was performed as described previously.[28] Western blots of CD43 immunoprecipitated products from DC total cell lysates were stained with HECA-452 mAb and rendered translucent by immersion in Hank's balanced salt solution (HBSS) with 10% glycerol, 10mM HEPES (pH 7.4) and 5mM $CaCl_2$. The blots were placed under a parallel-plate flow chamber, and Chinese hamster ovary (CHO) cells transfected with full-length human E-selectin cDNA (CHO-E) were perfused over blots at a shear stress of 0.17 dynes/cm^2. The number of interacting cells within and outside each stained region of the blot was videotaped for subsequent analysis. Mock-transfected CHO cells (CHO-M) were used to assess the specificity of the adhesive interactions on each stained region.

Enforced fucosylation of DCs by FTVII-treatment

Cells were treated with 0.07 mg/mL of fucosyltransferase VII (FTVII) (R&D systems) in HBSS containing 10 mM HEPES, 0.1% human serum albumin and 1mM GDP-fucose (Sigma-Aldrich), for 60 minutes at 37ºC. As negative control, cells were treated in the same buffer but without FTVII (buffer-treated). Cell viability was assessed routinely by trypan blue exclusion assay.

Microfluidic adhesion assay

To assess cell rolling, human umbilical vein endothelial cells (HUVECs) were grown to confluence in Bioflux microfluidic channels coated with 250µg/mL of human plasma fibronectin (Sigma-Aldrich). To stimulate the expression of E-selectin, HUVECs were incubated with 40 ng/mL of recombinant human tumor necrosis factor (TNF)-α (R&D), for 4 hours at 37ºC prior to use in the adhesion studies. DCs left untreated (buffer treated-BT) or FTVII-treated were suspended at a concentration of 10×10^6 cells/mL and perfused through the channels, at an initial shear rate of 0.5 dynes/cm^2; subsequently adjusted to shear stress ranging from 0.5 to 16 dynes/ cm^2. Controls for non-specific binding consisted of sialidase-treated DCs. All experiments were video-recorded for analysis and the number of rolling and arrested cells was determined.

Results

Glycoform of CD43, CD43E, is a functional E-selectin ligand on human APB mo-DCs

We have previously reported that CLA and HCELL are E-selectin ligands on human adult peripheral blood (APB) mo-DCs (Videira et al, unpublished data). In this study, we performed several biochemical studies and observed that, apart from CLA and HCELL, the glycoform of CD43 is also a functional E-selectin ligand expressed on the cell surface of mo-DCs obtained from APB.

Lysates of human APB mo-DCs resolved by SDS-PAGE and exposed by westernblot using E-selectin-Ig chimera (E-Ig) stained an E-Ig reactive ~120-130 kDa band, characteristic of CLA, and a ~80-90 kDa band, characteristic of HCELL (**Figure 1a**). Consistently, PSGL-1 immunoprecipitates from human mo-DC total cell lysates stained with E-Ig showed a ~120-140 kDa band, corresponding to CLA. However, after an exhaustive PSGL-1 immunoprecipitation (IP), confirmed by the absence of a PSGL-1-reactive band in the supernatant (Supn), an E-Ig reactive band continued to appear on the supernatant with the same molecular weight of PSGL-1 (**Figure 1b**). This suggests the presence of another sLe^X-scaffold protein in the cell lysates of human APB mo-DCs that co-migrates with PSGL-1 in reducing condition electrophoresis. Based on the electrophoretic mobility and since that the sLe^X-decorated glycoform of CD43 has been described to also migrate at ~120-130 kDa,[24] we checked for the presence of CD43 on mo-DCs by both flow cytometry and Western blot techniques. After confirming the presence of CD43 on mo-DC cell surface (**Figure 1c**), we performed a CD43 IP from human mo-DC total cell lysate and observed the presence of an E-Ig-reactive band on the CD43 immunoprecipitate product (**Figure 1d**).

To assess the E-selectin ligand activity of the sLe^X-glycoform of CD43 under physiologic flow conditions, we performed blot-rolling assays, on blots containing the CD43 immunoprecipitate product from APB mo-DC lysates. CHO-E rolling interactions were consistently observed on the CD43 band (**Figure 1e**). Specificity for E-selectin was demonstrated by the elimination of CHO-E cell rolling outside of band or by the lack of interaction on the band using CHO-mock cells (**Figure 1e**).

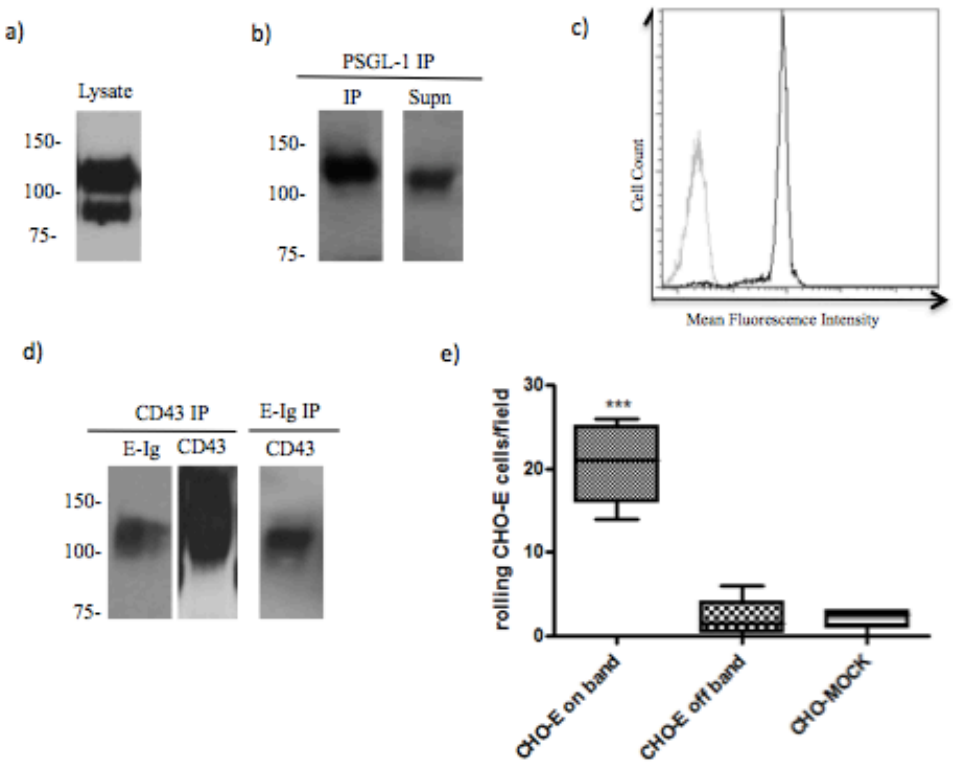

Figure 1. CD43 is functional E-selectin ligand on human mo-DCs from APB. a) *Western blot analysis of whole cell lysate of human ABP mo-DCs stained with E-Ig.* E-Ig staining of APB DC lysates resolved under reduced SDS-PAGE conditions revealed two principal bands at ~120-130 kDa and ~80-90 kDa. **b)** *Western blot analysis of PSGL-1 immunoprecipitation from human APB mo-DCs*. PSGL-1 was immunoprecipitated (IP) from human APB mo-DCs total cell lysate, resolved by SDS-PAGE, blotted, and stained with E-selectin-Ig chimera (E-Ig). "Supn" corresponds to the cleared lysate, i.e., following PSGL-1 immunoprecipitation. As shown, there is an E-Ig reactive band in the Supn lane, indicating the presence of another HECA-452-decorated glycoprotein that co-migrates with CLA. **c)** *Representative flow cytometry histogram of CD43 expression on human APB mo-DCs***.** Grey line represents incubation with isotype control and black line represents incubation with specific antibody. **d)** *Western blot analysis of E-Ig-reactivity of CD43.* CD43 proteins were immunoprecipitated from cell lysates of human ABP mo-DCs, resolved by SDS-PAGE, blotted, and stained with E-Ig or anti-CD43 antibody. In addition, E-selectin ligands were immunoprecipitated (E-Ig IP), resolved by SDS-PAGE, blotted, and stained with anti-CD43. Human APB mo-DCs express CD43 that display E-selectin reactive epitopes. **e)** *Blot rolling assay performed on immunoprecipitates of CD43 from mo-DCs.* CD43 immunoprecipitate was resolved by SDS-PAGE, blotted and stained with HECA-452 mAb. E-selectin transfected CHO cells (CHO-E) were perfused over blots at 0.17 dynes/cm^2.

Exofucosylation creates the E-selectin reactive glycoform of CD18 and new E-selectin-reactive glycoforms of CD44 on human APB mo-DCs

As previously demonstrated, α(1,3)-exofucosylation of cell surface glycans increases the expression of E-selectin ligands and thereby improves tethering and rolling properties of treated cells. (Videira et al., unpublished data; Silva et al., unpublished data) In particular, identification of cell surface glycoproteins capable of E-selectin binding following α(1,3)-exofucosylation allows for assessment of scaffold proteins that display sialylated lactosaminyl glycans, yet are not E-selectin ligands only because they lack relevant fucosylation.[13] We thus sought to determine whether exofocusylation would enforce the expression of new E-selectin ligands among human APB mo-DCs and which of the protein scaffolds are preferentially targeted by this enzymatic modification.

To this end, we treated human APB mo-DCs with the α(1,3)-fucosyltransferase FTVII and then performed an E-Ig immunoprecipitation (IP) from both FTVII treated and BT-treated human mo-DCs cell lysates; IP products were SDS-PAGE resolved and blotted with anti-HECA-452, anti-PSGL-1, anti-CD44 and anti-CD43. Western blot analysis of the E-Ig IP products from human mo-DC lysates showed a profound increase in HECA-452 reactivity following FTVII treatment (**Figure 2a**). This increased HECA-452 reactivity correlated specifically with the formation of E-reactive glycoforms of both CD43 and CD44 (**Figure 2a**). Interestingly, while FTVII exofucosylation created the standard isoform of HCELL (~80-90kDa), additional higher molecular weight variant isoforms of CD44 were created, as was observed by the formation of multiple high molecular CD44 reactive bands the in E-Ig product (**Figure 2a**). The glycoform of PSGL-1, CLA, was also increased after enforced FTVII exofocusylation, though only in a small amount relative to that of CD43E and HCELL. Surprisingly, another E-selectin reactive glycoprotein was created after enforced exofocusylation. As shown in **Figure 2b,** a CD18 reactive band appears in the E-Ig IP product from FTVII-treated moDC lysate lane with a molecular weight of ~80-90 kDa. This observation suggests the presence of sialylated lactosamine chains on CD18 scaffold that are accessible to FTVII reaction, resulting in the creation of the E-selectin reactive glycoform of CD18.

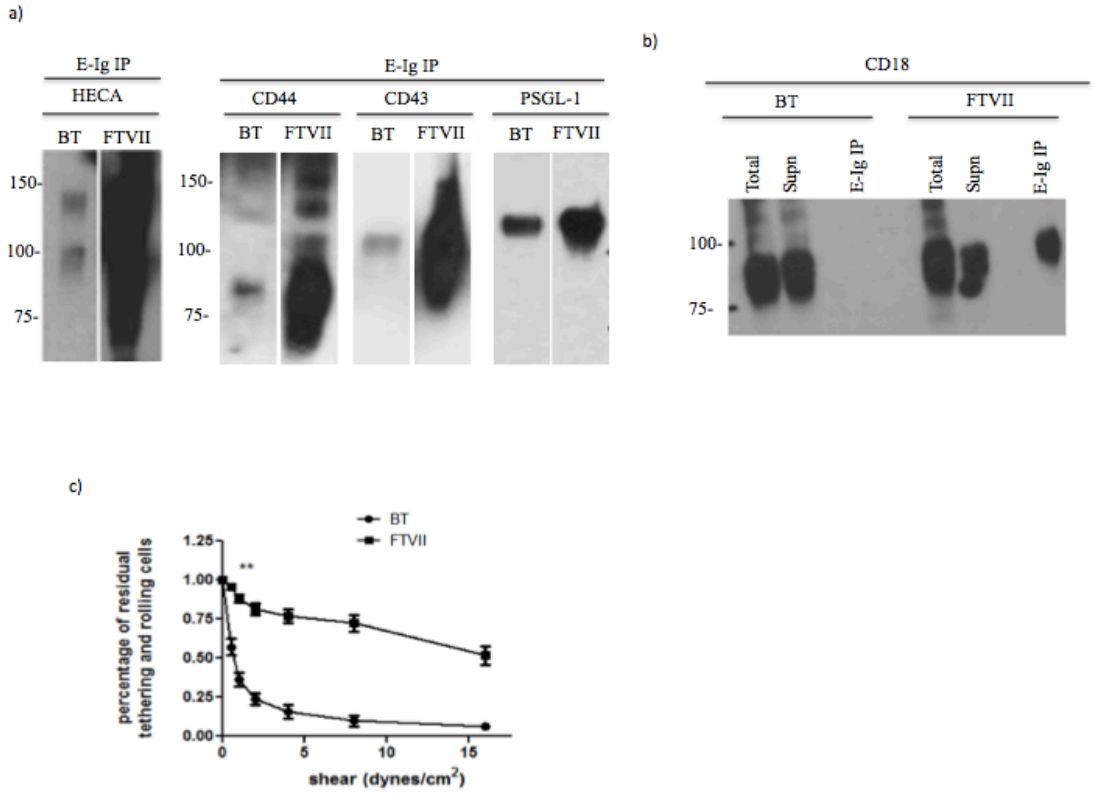

Figure 2. E-selectin reactive glycoform of CD18 is created after enforced exofucosylation. a) *FTVII-mediated exofucosylation of APB mo-DCs increases mainly HCELL and CD43E expression.* Human APB mo-DCs were FTVII-treated (FTVII) or Buffer treated (BT) and then lysated. Cell lysates were submitted to an E-Ig IP followed by SDS-PAGE and blotted with anti-HECA-452, anti-CD44, anti-CD43 and anti-PSGL-1 antibodies. A marked increase in E-Ig reactivity of the CD43 and CD44 scaffolds was observed, with a smaller increase in the E-Ig reactivity of PSGL-1. (**b)** *FTVII-mediated exofucosylation of APB mo-DCs creates a novel E-selectin binding glycoform of CD18.* E-Ig immunoprecipitation was performed on cell lysates from FTVII-treated and BT mo-DCs. Total cell lysate (Total), immunoprecipitate (IP), and cleared lysate (Supn) were resolved by SDS-PAGE and blotted with anti-CD18 mAb. CD18-reactivity on E-Ig IP lane was observed, indicating enforced expression of an E-selectin ligand glycoform of CD18. ***c)*** *Functional E-selectin ligands can be formed after FTVII reaction***.** Parallel plate flow chamber analysis of tethering and rolling interactions of buffer treated (BT) and FTVII-treated mo-DCs (FTVII) on TNF-α-activated HUVECs. FTVII treatment of APB mo-DCs significantly augmented their ability to tether and roll under shear stress conditions.

To further analyse the effect of FTVII treatment, we assessed the ability of human APB mo-DCs to bind to TNF-α-stimulated HUVEC monolayers under physiological shear stress conditions. BT-mo-DCs exhibited a modest capacity to tether and roll, whereas, FTVII treatment led to a significant increase in the number of tethering/rolling mo-DCs, and of firmly adherent cells (**Figure 2c**). These data suggest that the E-selectin-binding glycoforms of CD43 and CD44, as well the new-formed E-selectin reactive CD18 glycoform, may play an important role in E-selectin binding after FTVII reaction.

Localization of E-selectin ligands at cell surface of human APB mo-DCs

We next determinated the localization pattern of E-selectin ligands on the cell surface of human mo-DCs. Human monocytes were cultured on poly-lysine coated plates in the presence of GM-CSF and IL-4 for 7 days. After differentiation into mo-DCs, these cells were co-stained with FITC-conjugated E-Ig and rhodamine-conjugated phalloidin. As shown in **Figure 3**, E-selectin ligand distribution (green) is predominantly coincident with F-actin localization (red), particularly with dendritic cell protrusions. The co-distribution of E-selectin ligands and F-actin in human mo-DCs suggests that E-selectin reactive determinants are mainly localized to the migration front of these cells.

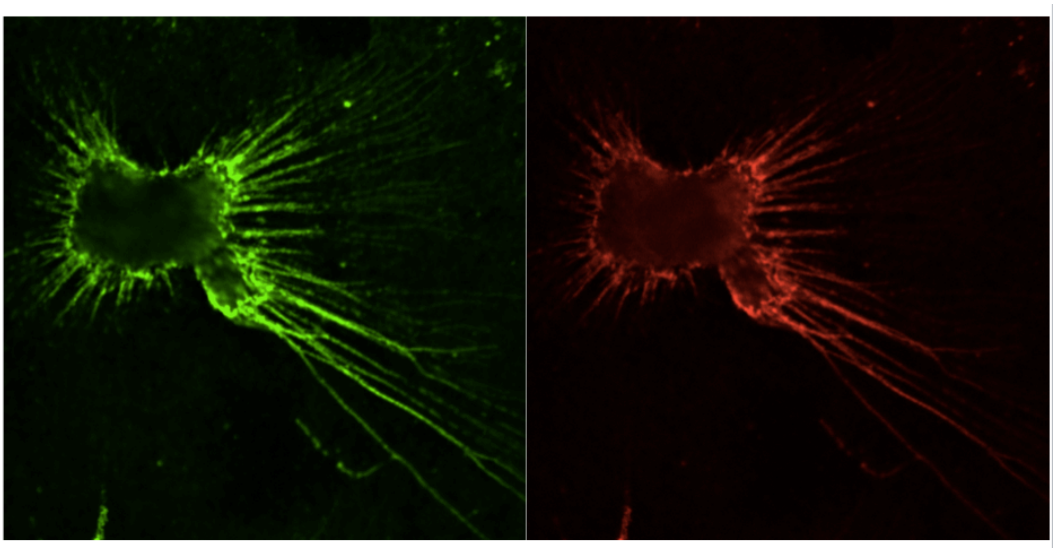

Figure 3. Immunostaining of human mo-DCs shows co-localization of E-selectin ligand reactivity and F-actin staining. Human mo-DCs were double labeled with E-Ig, for E-selectin ligands (green), and rhodamine-conjugated phalloidin (red) for F-actin. Images of slices were obtained in a Leica TCS SP2 AOBS confocal microscope and assembled with Leica Confocal Software.

Umbilical cord blood monocytes derived-DCs do not express any glycoprotein with E-selectin binding activity

The characterization of E-selectin ligand expression on DCs derived from human APB monocytes opened new insights into the understanding of DCs migratory functionality. However, little is known regarding E-selectin ligands expressed by DCs procured from other human cellular sources. There is emerging interest in the development of new methods capable to overcome the limitations in current protocols used for therapeutic DC-vaccines.

Monocytes obtained from umbilical cord are currently being studied to investigate their potential as an alternative DC source. Thus, we sought to analyze the E-selectin binding pattern of mo-DCs derived from umbilical cord blood (UCB). For this purpose, we examined their E-selectin binding reactivity by Western blot and flow cytometry analysis, using E-Ig as a probe, as it binds to sialofucosylated E-selectin binding determinants. Unexpectedly, and in contrast to APB mo-DCs, Western blot analysis revealed that these cells do not express glycoproteins with E-selectin binding activity, as shown by the absence of E-Ig reactivity (**Figure 4a**). Interestingly, by flow cytometry the level of E-Ig reactivity was positive (**Figure 4b**), suggesting that human UCB mo-DCs express E-selectin binding determinants mainly displayed on cell surface via glycolipid scaffolds. The distinct E-selectin ligand expression pattern between UCB and APB mo-DCs prompted us to analyze the E-Ig reactivity of monocytes, the mo-DC precursors. Intriguingly, monocytes from umbilical cord blood showed strong E-Ig reactivity by westernblot analysis, staining two E-selectin reactive bands at ~120-130 kDa band and ~80-90 kDa. Yet, E-Ig reactivity was slightly lower compared to APB monocytes (**Figure 4c**). This data suggests that the E-selectin ligand glycoprotein expression on UCB mo-DCs is lost during the DC differentiation and is not intrinsic to UCB monocytes.

We next assessed whether enforced α(1,3)-fucosylation would increase surface expression of E-selectin ligand glycoproteins on UCB mo-DCs. After FTVII reaction, westernblot analysis showed that mo-DCs from UCB exhibit a marked increase in expression of glycoproteins with E-selectin binding activity. Distint bands were revealed with molecular weights corresponding to CLA/CD43E (~120-140 kDa) and HCELL (~80-90 kDa) (**Figure 4d**). The expression of glycoproteins E-selectin binding reactivity after exofocusylation shows that UCB mo-DCs natively express unfocusylated sialyllactosamine units on protein scaffolds. Therefore, the lack of E-selectin ligand determinants on protein scaffolds is potentially due to an insufficient activity of α(1,3)-fucosylatransferases and/or higher activity of α(1,3)fucosidases.

To fully assess the impact of enforced exofocusylation, BT-treated or FTVII-treated UCB mo-DCs were perfused over TNF-α stimulated HUVEC under physiologic shear stress. As shown in **Figure 4e,** FTVII-treated UCB mo-DCs maintained high levels of rolling and tethering contacts with HUVEC monolayer at high levels of shear resistance. In contrast, BT-

treated UCB mo-DCs displayed a dramatically decreased ability to tether and roll at shear stress levels higher than 1 dynes/cm^2.

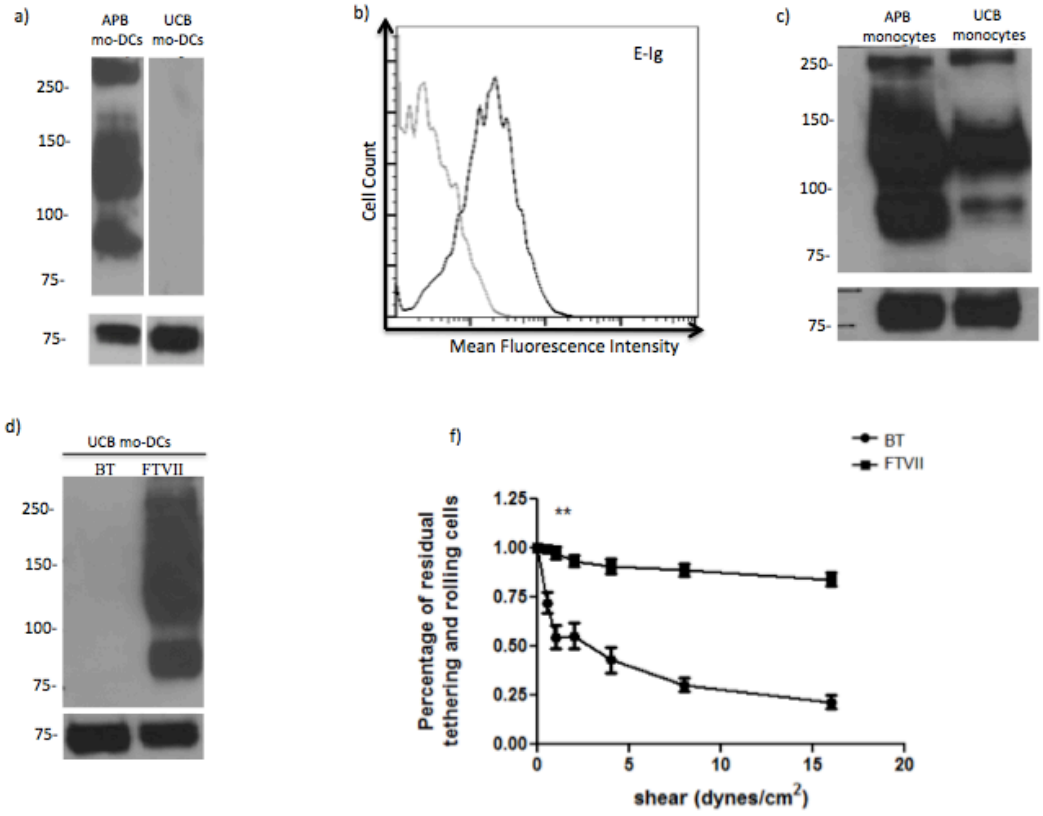

Figure 4. Mo-DCs obtained from UCB do not express glycoproteins with E-selectin binding reactivity. a) *Western blot analysis of E-Ig-reactive proteins of human APB and UCB mo-DCs*. Whole cell lysates from APB and UCB mo-DCs were resolved by SDS-PAGE and blotted with E-Ig chimera. ABP mo-DCs show high E-Ig reactivy, whereas UCB mo-DCs lack E-Ig staining. CD44 staining (molecular weight ~80-90kDa) was used as protein loading control. **b)** *Representative flow cytometry histogram of E-Ig staining of UCB mo-DCs*. Grey line represents E-selectin binding in the absence of Ca^{2+} (EDTA) and black line represents incubation with E-Ig. As shown, human UCB mo-DCs show E-Ig reactivity by flow cytometry **c)** *Western blot analysis of whole cell lysate of human APB and UCB monocytes stained with E-Ig.* E-Ig staining of both APB and UCB monocyte lysates resolved under reduced SDS-PAGE conditions revealed strong E-Ig reactivity. CD44 staining (molecular weight ~80-90kDa) was used as protein loading control. **d)** *Western blot analysis of E-Ig reactivity of UCB mo-DCs that were buffer treated (BT) or FTVII-treated (FTVII)*. FTVII-mediated exofucosylation of UCB mo-DCs creates E-selectin ligands on multiple cell surface glycoproteins. CD44 staining (molecular weight ~80-90kDa) was used as protein loading control. **e)** *FTVII treatment creates functional E-selectin ligands on UCB mo-DCs.* Human UCB mo-DCs treated with FTVII (FTVII) or buffer alone (BT) were perfused into a parallel-plate flow chamber seeded with TNF-α-stimulated HUVECs. FTVII-treated cells exhibited a significantly increase of tethering and rolling contacts.

Murine DCs do not express the E-selectin binding glycoform of CD44

To determine the E-selectin ligand expression pattern on murine DCs, we analysed by western blot and flow cytometry two types of DCs: bone-marrow derived DCs (BM-DCs) and splenic DCs (sDCs). Interestingly, these two types of murine DCs showed different E-selectin ligand reactivity patterns between each other.

Murine BM cells were isolated from bone marrow and grown for either 5 or 8 days in the presence of GM-CSF, allowing differentiation of BM precursors into DCs. After differentiation, DCs typically express a combination of cell surface markers, such as CD11c, major histocompatibility complex class II (MHC-II) and CD11b (myeloid marker). In cultures growing for 5 days, we typically observed a differentiation of BM precursors into DCs of about 55% and these cells were characterized by a high expression of CD11b and low expression of MHC-II, suggesting an immature phenotype, whereas in cultures growing for 8 days, around 70% of the cells were $CD11c^{+}$, $CD11b^{high}$ and MHC-II^{high}, suggesting a mature phenotype**.**

Although, murine BM-DCs showed a strong E-Ig and HECA-452 reactivity by flow cytometric analysis (**Figure 5a**), our western blot results suggest that these cells don't express any glycoproteins with E-selectin binding activity, as evidenced by the absence of E-Ig reactivity at both culture time periods assessed (**Figure 5b**); even though these cells express a signifivant amount of protein scaffolds, such as CD44 and PSGL-1 (data not shown). These data indicate that the E-selectin ligand epitopes expressed on murine BM-DCs are prominently displayed in glycolipids. Interestingly, when we analyzed by flow cytometry the expression of E-selectin ligands at day 5 and 8, we observed that E-selectin ligand expression tend to decrease over time, i.e BM-DCs grown for 5 days exhibited a higher expression of E-selectin ligands than those grown for 8 days (**Figure 5a**).

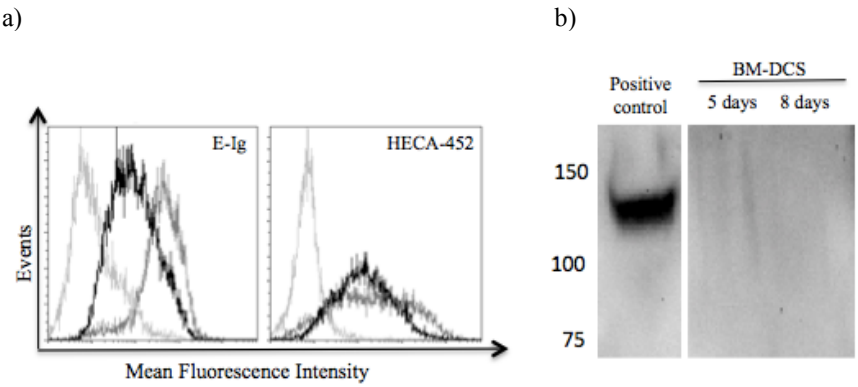

Figure 5. Murine BM-DCs do not express glycoproteins with E-selectin ligand activity. a) *Flow cytometry analysis of E-selectin ligand expression on murine BM-DCs assessed by E-Ig and HECA reactivity*. Black lines represent E-Ig or HECA reactivity of BM-DCs cultured for 5, whereas, darker grey lines represent E-Ig or HECA reactivity of BM-DCs cultured for 8 days. Lighter grey lines represent isotype control (or E-selectin binding in the absence of Ca^{2+}). BM-DCs show considerable E-Ig and HECA reactivity that declines with increased culture time period. **b)** *Western blot analysis of HECA-452 reactivity of whole cell lysates from murine BM-DCs*. BM-DCs lack HECA-reactive glycoproteins.

By contrast, sDCs showed a strong HECA-452 reactivity by both flow cytometry (**Figure 6a**) and western blot (**Figure 6b**) analysis. Westernblots of total sDCs cell lysate blotted for HECA-452 showed a reactive ~120-130kDa band. Due to the molecular size of this HECA-reactive band, we immunprecipitated PSGL-1 from murine sDCs total lysate and stained with HECA-452. Westernblots of E-Ig immunoprecipitated products followed by PSGL-1 staining were also performed (**Figure 6b**). In both cases, we confirmed that the HECA-452 reactive-band present on whole cell lysate of sDCs corresponds to the glycovariant of PSGL-1, CLA (**Figure 6b**).

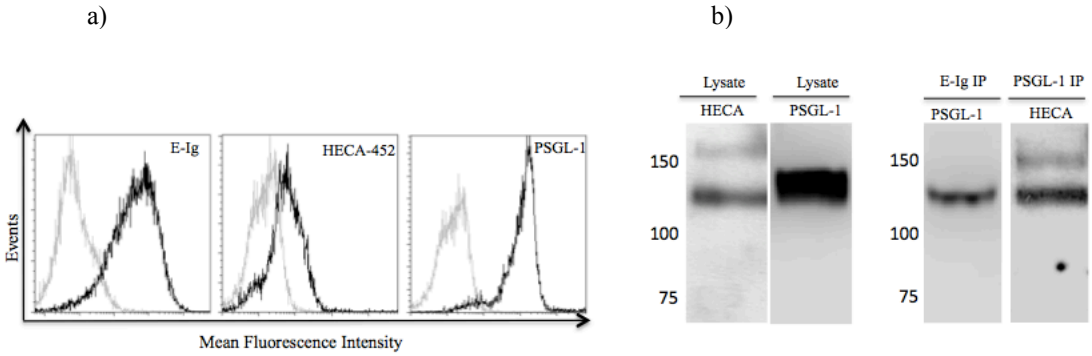

Figure 6. CLA is expressed by murine splenic DCs. a) *Flow cytometry analysis of E-Ig, HECA-452 and PSGL-1 expression on murine sDCs.* Grey lines represent isotype control (or E-selectin binding in the absence of Ca^{2+}) and black lines represent incubation with specific antibodies or E-Ig. Murine sDCs express high levels

of E-Ig, HECA and PSGL-1. **b)** *Identification of glycoprotein E-selectin ligands expressed by murine sDCs*. *Left*: Western blot analysis of whole cell lysate from murine spleen DCs. Total cell lysates were resolved by SDS-PAGE and blotted with anti-HECA-452 or anti-PSGL-1 antibodies. *Right*: E-selectin ligands and PSGL-1 from murine sDCs were immunoprecipitated and then resolved by SDS-PAGE and blotted with PSGL-1 and E-Ig, respectively. Western blots reveal expression of the E-selectin ligand CLA on sDCs.

Discussion

Dendritic cells (DCs) are highly efficient antigen-presenting cells that play a key role in the induction of immunity.[29] Due to their ability to elicit both innate and adaptive immune responses, DCs have been exploit as a powerful toll in anti-cancer vaccines during the last two decades.[30] Although DC-based vaccines are able to induce tumor-specific immune responses in mice models, limited progress has been shown in clinical trials, since they are ineffective at inhibiting tumor growth in treated cancer patients.[31] Thus, new strategies are urgently needed to overcome the poor objective clinical response observed. It is well established that tumor microenvironment is rich in tumor infiltrating effector memory T cells, therefore, directing DCs to these tissues would enhance local antigen presentation and induce a stronger and quicker cellular-mediated anti-cancer immune response.[9] Since tumor microvasculature highly expresses E-selectin and the recruitment of circulating leukocytes to these sites is mainly mediated by binding to vascular E-selectin,[13] we sought to perform a detailed characterization of the E-selectin ligands that are expressed by human and mouse DCs, obtained from different cellular sources, and develop methodologies that could up-regulate the expression of these molecules. Our results opened new insights into species-specific and cellular sources-specific differences in the expression of E-selectin ligands by DCs, providing a novel understanding on DCs migration to sites of inflammation/ tumor in mice and humans.

Human DCs can be generated from different cellular sources, however the most common approach used, in immunotherapy, is the differentiation of DCs from monocytes.[32] These are normally obtained from peripheral blood, but recently there has been an increasing interest in monocytes obtained from umbilical cord blood sources.[33] In this study, we first analyzed the E-selectin reactive determinants displayed in the protein scaffolds of both adult peripheral blood (ABP) and umbilical cord blood (UCB) monocyte-derived DCs (mo-DCs). Our data show that human APB mo-DCs display a large set of glycoprotein E-selectin ligands, in contrast to UCB mo-DCs. Previously, we reported that APB mo-DCs express the sLe^X-

decorated form of PSGL-1, CLA, and the E-selectin reactive glycoform of CD44, HCELL. (Videira et al., unpublished data) Here, our data is the first to show that the glycoform of CD43, CD43E, is also an E-selectin ligand expressed by APB mo-DCs. Moreover, in blot rolling assays of CD43 immunoprecipitates from APB mo-DC lysates, CD43 strongly supported E-selectin-specific interactions, suggesting that CD43E is a functional E-selectin ligand on APB mo-DCs. CD43 is a surface sialomucin expressed exclusively on hematopoietic cells[34] and has previously been described to function as an E-selectin ligand on both human[24] and murine[25,35] T cells, on human $CD34^+$ and mouse LSK cells,[36] on murine neutrophils[37] and on human monocytes (Silva et al., unpublished data). Notably, immunofluorescence studies show that the E-selectin binding epitopes are distributed prominently to the migrating front of APB mo-DCs, with particular relevance for the veil like-cell actin-rich protrusive structures. In amoeboid cells, like DCs, migration is highly dependent on cortical actin cytoskeletal tension, therefore co-localization of E-selectin ligands with F-actin filaments may facilitate interaction of E-selectin ligands with E-selectin on endothelial surfaces.[38,39]

On the other hand, we observed that UCB mo-DCs do not natively express any glycoprotein with E-selectin ligand activity. Nevertheless, UCB mo-DCs show E-selectin binding activity by flow cytometry, which suggests that these cells have E-selectin binding determinants displayed in glycolipid scaffolds. While E-selectin binding determinants can be displayed via protein or lipid scaffolds, glycoproteins play a major role in the initial contact to E-selectin under hemodynamic shear stress. Glycolipids are significantly smaller than glycoproteins, and therefore not accessible to E-selectin as glycoproteins.[40,41] Thus, our data may suggest a malfunctioning ability of UCB mo-DCs to migrate to inflamed tissues since they lack E-selectin reactive glycoproteins. Indeed, our finding is in agreement with previously studies reporting that UCB cells display a poor ability to migrate to bone marrow, where E-selectin is expressed constitutively.[42,43]

To assess whether we could augment the DC cell surface expression of E-selectin ligands, and thereby, increase their ability to migrate to inflamed tissues, we performed $\alpha(1,3)$-exofucosylation of both APB and UCB mo-DCs and analyzed their E-Ig reactivity and ability to bind to E-selectin under hemodynamic flow conditions. Enforced exofucosylation markedly induces E-selectin binding activity in both types of mo-DCs, as assessed by western blot technique. In agreement, the ability of these cells to bind to TNF-α stimulated HUVECs

under shear stress conditions is also dramatically enhanced. Notably, enforced fucosylation of APB mo-DCs, not only induces increased expression of functional CLA, HCELL and CD43E, but also the newly formed E-selectin reactive glycoform of CD18 and high molecular weight glycoforms of HCELL. So far, the E-selectin glycoform of CD18 had only been described on neutrophils,[44] while the high molecular weight E-selectin reactive glycoforms of CD44 had only been described in certain types of tumor cells.[45] The enhanced ability of FTVII-treated APB mo-DCs to tether and roll under hemodynamic flow conditions compared to untreated cells (BT) highlight the role of the newly formed glycoforms of CD18 and CD44 as potent E-selectin ligands. Concerning the exofucosylation of UCB cells, the creation of sLe^X determinants indicates that these cells natively express sialylated lactosamine units, which can be extrinsically fucosylated. Therefore, the lack of E-selectin reactive glycoproteins on UCB mo-DCs is due to the lack of fucose residues within the sialylated lactosamine units, which can be overcome by an exofucosylation reaction step.

Striking differences related to the E-selectin binding profile were also observed between murine bone marrow derived DCs (BM-DCs) and murine splenic DCs (sDCs). Similar to human UCB mo-DCs, murine BM-DCs are devoid of E-selectin reactive glycoproteins, but not of E-selectin binding glycolipids, while sDCs strongly express CLA. Interestingly, BM-DCs that were grown for 5 days exhibited a higher expression of E-selectin ligands determinants, as assessed by flow cytometry, than those grown for 8 days. BM-DCs cultured for 5 days were not fully differentiated and exhibit a more immature phenotype. Thus, these observations suggest that a higher expression of E-selectin ligand epitopes are associated with an immature and incomplete differentiation phenotype of murine BM-DCs. Also, it is striking that any of these murine DCs express the E-selectin reactive glycoform of CD44, HCELL, which is the most potent E-selectin ligand described so far.[36] Indeed, HCELL has only been described in human cells, while the others E-selectin reactive glycoproteins reported (CLA and CD43E) have been described to function as E-selectin ligands in both human and murine cells.[13]

Collectively, our findings disclose critical and undiscovered cellular sources-specific and species-specific diversity in the expression of E-selectin ligand glycoproteins among DCs. Our data suggest that monocytes isolated form adult peripheral blood may be a better source for *ex vivo* generated anti-cancer DC-based vaccines since APB mo-DCs natively display high amounts of E-selectin ligands, which are required for DC migration to inflamed/tumoral tissues. On the other hand, UCB mo-DCs may be therapeutically valued in transplantation or

autoimmune disease contexts. Finally and similar to previous studies,[36,41] we demonstrate that exofucosylation is a powerful platform technology for enforcing E-selectin ligand expression, which would promote the trafficking of DCs to sites of inflammation/tumor in the body.

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Authorship Contributions and Disclosure of Conflicts of Interest

The study was conceived by R.S., who designed and supervised all research, funded the research and wrote the manuscript. P.V. and C.T designed and supervised research, funded the research and wrote the manuscript. M.S. and C.D. designed and performed research, collected and analyzed data, and wrote the manuscript. According to the National Institutes of Health policies and procedures, the Brigham & Women's Hospital has assigned intellectual property rights regarding HCELL to the inventor (R.S.), who may benefit financially if the technology is licensed. R.S.'s ownership interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policy. All other authors declare no competing financial interests.

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Chapter 6

Sialic acid removal from dendritic cells improves antigen cross-presentation and boosts anti-tumor immune responses

Paper IV- ***Submitted to Oncotarget***

TITLE

Sialic acid removal from dendritic cells improves antigen cross-presentation and boosts anti-tumor immune responses

RUNNING TITLE

Desialylated DCs boost anti-tumor imune responses

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KEY POINTS:

1. **Sialic acid removal from DC surface elicits proliferation of T cell and their polarization towards Th1 phenotype**
2. **Desialylation of DCs induces cross-presentation of tumor antigens via MHC-I to $CD8^+$ T cells**

Abstract

Dendritic cells (DCs) hold promise for anti-cancer immunotherapy, as they initiate strong and long-lived specific T cell responses. However, at the clinical setting, their efficiency is limited and the identification of strategies to improve DC responses is critically needed. Sialic acids have a potential immunomodulatory role due to their exposed location at the termini of cell surface glycans. Human DCs have a high content of sialic acids, which influences their maturation and co-stimulation capacity. Here, we aimed to understand whether exogenous desialylation, obtained by sialidase treatment, improves DC-mediated anti-tumor immune responses. Compared to fully sialylated DCs, the desialylated human DCs, loaded with whole tumor cell antigens showed enhanced ability to induce autologous T cells to proliferate, to secret Th1 cytokines, and to specifically kill tumor cells. Desialylated human DCs showed an increased expression of MHC class I and II and co-stimulatory molecules and secretion of IL-12. Desialylated HLA-A*02:01 DCs pulsed with synthetic long or short peptides from the tumor antigen gp100 displayed enhanced antigen cross-presentation or peptide binding to MHC-I, as evaluated based on the activation of $gp100_{280–288}$ specific $CD8^+$ cytotoxic T cells. To evaluate whether the effect was restricted to human DCs, we also evaluated murine DCs. Concordantly, desialylated murine DCs displayed higher MHC class I and II and co-stimulatory molecules and antigen cross-presentation on MHC-I. These DCs showed higher ability to activate antigen-specific $CD4^+$ and $CD8^+$ T cells, to specifically kill antigen-expressing melanoma cells. Taken together, our data demonstrates that desialylation improves the capacity of DCs to elicit T cell-mediated anti-tumor activity. This improvement is related with enhanced antigen presentation through MHC-I due to increased MHC-I expression and binding of antigen to MHC-I.

This study offers key insights to optimize the capacity of DCs to boost anti-tumor immunity, and indicates that treatment with an exogenous sialidase is a potential technology to improve the efficacy and applicability of antigen loaded-DC-based cancer vaccine.

Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells of the immune system and thus critical to initiate anti-tumor immune responses. Several studies have shown the potential of these cells as therapeutic vaccination against cancer.[1-4] However, only a few vaccines have been approved for clinical use.[5] One of the challenges has been the establishment of efficient approaches that enhance the maturation profile of DCs and in particular the presentation of antigens to cytotoxic T cells.[6] In fact, efficient induction of anti-tumor responses, requires that DCs undergo proper maturation, characterized by: increased expression of major histocompatibility complex (MHC) molecules, needed to present antigens to T cells; increased expression of co-stimulatory molecules, such as CD80 and CD86, to promote DCs interaction with T cells;[7] and secretion of pro-inflammatory cytokines, such as the Th1-inducing cytokine interleukin (IL)-12, required for anti-tumor activity.[8] Importantly, endogenous antigens are presented through MHC-I molecules to $CD8^+$ cytotoxic T cells, after proteasomal degradation, while exogenous antigens are classically presented via MHC-II to $CD4^+$ helper T cells, after processing via the endolysosomal route.[2] DCs are also endowed with unique antigen processing pathways that enable exogenous antigens to escape endolysosomes and then enter proteasomal degradation to be presented in the context of MHC-I molecules, in a process called cross-presentation.[9,10] Furthermore, exogenously supplied high affinity peptides can bind directly to MHC-I, without previous endocytosis and processing.[11] Since most tumor-associated antigens used for DC vaccination are of exogenous origin, strategies that foment antigen presentation through MHC-I are crucial for the generation of $CD8^+$ cytotoxic T cells response, and thereby for the generation of an anti-tumor immune response.[10,12,13]

Glycans that decorate cell membrane proteins play a key role in the modulation of cell-cell and receptor-ligand interactions in several immunological processes.[14-16] The terminal positions of these glycans may be occupied by a negatively charged and non-reducing monosaccharide, named sialic acid.[17] Due to this exposed location, sialic acids are involved in many immunological functions. This includes the regulation of the immune cell activation through sialic acid recognition by Siglecs, a family of inhibitory sialic acid–dependent receptors[18] and sialic acid recognition by certain microbes upon infection.[19,20] Sialic acid may also alter the biophysical properties of cellular interactions by providing negative charge repulsion between cells, and therefore mask or prevent recognition by other receptors such as galectins.[21,22] The sialic acids content at cell surface is tightly regulated, differing among cell

types and varying markedly with regard to cell differentiation and stimuli.[23,24]

In previous studies, we demonstrated that the surface of human monocyte-derived DCs (MoDCs) is highly sialylated mainly due to the enzymatic activity of the sialyltransferases ST3Gal-I and ST6Gal-I, which contribute to the α(2,3)- and α(2,6)-sialic acid transfer to cell surface glycoconjugates, respectively. The removal of sialic acids by sialidase treatment induces DC maturation.[24] Consistently, desialylated MoDCs loaded with toxoid antigen have increased ability to activate autologous T cells.[25] Our observations suggest that the sialic acids present at cell surface of DCs contribute to dampen DC maturation and further ability of DCs to activate T cells. Yet, the role of cell surface sialic acids in DC immunogenicity against tumor cells is still unknown.

Here, we first sought to investigate whether DCs generated by well-established methods employed in clinical trials (e.g., NCT01042366, NCT00683241, and NCT00442754) had improved anti-tumor immune responses when treated with sialidase. Our data show that sialidase treatment of human MoDCs, loaded with whole tumor cell antigens, improves their ability to specifically activate autologous T cells and consequently, enhances T cells' ability to kill tumor cells. We further assessed the cellular effects of sialidase treatment and observed these MoDCs showed increased antigen-presenting and co-stimulatory abilities, resulting in higher polarization of T cells towards a Th1 phenotype. Murine splenic DCs (sDCs) also showed a similar improved capacity to induce anti-tumor immune responses when treated with sialidase. Notably, both MoDCs and sDCs showed increased cross-presentation of antigens through MHC-I to $CD8^+$ T cells. These results indicate that desialylation of DCs represents an efficient and powerful tool to elicit tumor-specific immune response, with potential impact in DC-based anti-tumor immunotherapy.

Materials and Methods

Reagents

Culture media, RPMI-1640, DMEM and IMDM, fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate, trypsin-EDTA, L-glutamine and glutamax were purchased from Gibco (Grand Island, NY, USA). Recombinant human granulocyte-macrophage colony-

stimulating factor (GM-CSF), IL-2 and tumor necrosis factor (TNF)-α, and the cell isolation immunomagnetic beads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant human IL-4 was purchase from R&D Systems (Minneapolis, MN, USA). The CellTrace™ CFSE Cell Proliferation Kit was purchased from Molecular Probes, and annexin V from Immunotools (Friesoythe, Germany).

The following anti-human antibodies (mAbs) were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD1a from BD Biosciences (San Jose, CA, USA); phycoerythrin (PE)-conjugated anti-CD1c from BioLegend (San Diego, CA, USA); allophycocyanin (APC)-conjugated anti-HLA-DR from Immunostep (Salamanca, Spain); and APC conjugated anti-CD3, anti-CD4; peridinin chlorophyll protein (PercP)-conjugated anti-CD8; PE–conjugated anti-CD14, anti-CD40, anti-CD69 and anti-CD80; and FITC-conjugated anti-CD45 and anti-CD86 mAbs from Immunotools.

The following anti-mouse mAbs were used: FITC-conjugated anti-CD11c, APC-conjugated anti-B220, anti-CD11b, PercP-conjugated anti-CD8α, PE-conjugated anti-IA^b and anti-CD86 mAbs from BD Biosciences; PercP-conjugated anti-CD4, FITC-conjugated anti-H-$2K^b$, biotin-conjugated CD80 and PE-conjugated anti-SIINFELK/H-2Kb mAbs from eBioscience (San Diego, USA); PE-conjugated streptavidin (Biolegend) was used in combination with biotin-conjugated anti-CD80 mAb. Unless otherwise stated, remaining reagents and media were purchased from Sigma Chemical Co. (Sigma, St Louis, MO, USA).

Flow cytometry

Cell surface staining was assessed by using fluorescence-conjugated or unlabeled mAb followed by fluorescently-conjugated secondary mAb, according to manufacturer instructions. For characterization of murine splenic DCs (sDC), blockade of nonspecific binding was performed by pre-incubation with 1% of anti-mouse CD16/32 (eBioscience) for 30 min at 4ºC. Data was acquired using either an Attune Acoustic Focusing Cytometer (Applied Biosystems, USA) or a FACSCalibur Cytometer (Becton-Dickinson, FranklinLakes, NJ, USA) and data analysed using either the Attune Cytometric Software v2.1 or the FlowJo software version 10.0.5 (TreeStar, San Carlos, CA, USA).

Human tumor cell line culture and preparation of whole cell lysates

The HLA-A*02:01 positive breast cancer cell line MCF-7 was originally isolated from a pleural effusion of a 69-year-old caucasian woman with a diagnosis of invasive breast ductal carcinoma.[26] The MCF-7-GFP is a MCF-7 variant that constitutively expresses the green fluorescent protein (GFP). Both cell lines were obtained from American Type Culture Collection and routinely cultured in complete DMEM media: DMEM medium with 10% FBS, 2 mM L-glutamine, and 100 μg/mL penicillin/streptomycin.

To obtain cell lysates as source of antigens, cells were grown until 80% confluence, collected, washed and resuspended in RPMI medium at a concentration of 5×10^6 cells/mL. Cells were lysed through four freeze (-80 ºC) and thaw (37 ºC) cycles and the cell debris removed by centrifugation. Complete cell lysis was confirmed microscopically by trypan blue and by culture test. The protein concentration of the tumor cell lysates was determined using Pierce BCA Protein Assay kit (Thermo Scientific; Germany)

Generation of human MoDCs

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of human peripheral blood from healthy volunteers provided by the Portuguese Blood and Transplantation Institute, after informed consent and approval by institutional ethical committee. Monocytes were enriched using anti-CD14 mAb-coated immunomagnetic beads. The $CD14^+$ cells were cultured at a concentration of 1×10^6 cells/mL in RPMI 1640 medium, 10% FBS, 2 mM L-glutamine, 100μg/mL penicillin/streptomycin, 1% pyruvate and 50 μM 2-mercaptoethanol (complete RPMI medium) supplemented with 750 U/mL of IL-4 and 1000 U/mL of GM-CSF, as described previously.[27] After 5-7 days in culture, the generation of MoDC, was confirmed by flow cytometry analysis.

Mice

Six to eight weeks old C57BL/6 (B6) mice, and the B6 transgenic mice OT-I (producing OVA-specific MHC-I restricted $CD8^+$ T cells) and OT-II (producing OVA-specific MHC-II restricted $CD4^+$ T cells) were housed and bred at the animal facility of Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal). Mice were sacrificed by carbon dioxide inhalation

followed by cervical dislocation. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC), in agreement with the Federation of European Laboratory Animal Science Associations (FELASA) directives, approval ID number AO10/2010.

Murine cell culture

The mouse melanoma cell line B16, transfected with the FMS-like tyrosine kinase 3 ligand gene (B16-Flt3-L) was gently provided by Dr. J. Lafaille (Skirball Institute of Biomolecular Medicine, NYU, USA) and cultured in complete RPMI medium. The B16 cell line transfected with the ovalbumin gene (B16-OVA) was gently provided by Dr. L. F. Moita (IMM, Lisboa, Portugal) and was grown in complete DMEM medium. Cells were split approximately every three days, after cell detachment using trypsin/EDTA.

Generation of murine DCs

To generate large numbers of splenic DCs (sDCs), female C57BL/6 mice were injected subcutaneously in the flank with 2×10^5 B16-Flt3-L cells, as described.[28] After 10 to 15 days, mice were sacrificed and splenocytes isolated by mechanical disruption of spleens and filtered. After red blood cell lysis (150 mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA), the sDCs were further sorted by positive immunomagnetic selection using anti-CD11c mAb-coated immunomagnetic beads following manufacturer´s instructions. The isolated cell fraction was typically ~ 97% $CD11c^+$ cells. Further characterization of DC subsets was performed (**Supplementary Figure S1**).

T cell isolation and culture

The human T cells used in this work comprised the $CD14^-$ cell fraction of PBMCs (> 70% $CD3^+$ cells), obtained after immunomagnetic isolation of monocytes ($CD14^+$), described above. For cytotoxicity experiments, T cells were further enriched using anti-CD3 mAb-coated immunomagnetic beads (> 97% $CD3^+$ cells). The $CD8^+$ T cells transduced with a TCR gene specific for the HLA-A*02:01 restricted $gp100_{280–288}$ peptide were cultured as

described.[29] Murine T cells were obtained from splenocytes from B6.OT-I and B6.OT-II TCR transgenic mice, obtained by spleen mechanical disruption, followed by red blood cell lysis, as described above.

Antigen loading and stimulation of DCs

For loading MoDCs with tumor antigen, MoDCs were incubated with protein lysate (1mg per 5×10^6 MoDCs, per mL), in complete RPMI medium, for 4h at 37 ºC. When needed, MoDCs were stimulated for 24h with TNF-α (1000 IU/mL).

For loading sDCs with OVA, sDCs were incubated with OVA (0.1mg per 0.5×10^6 sDCs, per mL), in complete RPMI medium, for 4-6h at 37ºC.

Sialidase treatment

Cells were treated with sialidase from *Clostridium perfringens* (Roche Diagnostics, Basel, Switzerland) at a concentration of 0.1 U/mL per 50×10^6 cells in 50 mM sodium phosphate buffer (pH 6.0) or RPMI medium for 60 min at 37ºC. Treatment was stopped by diluting 10 times the reaction volume with complete RPMI medium. The efficacy of sialidase treatment was confirmed by lectin staining with FITC-labeled *Sambucus nigra* lectin (SNA), -*Maackia amurensis* lectin II (MAA) and -*Peanut agglutinin lectin* (PNA) (Vector Laboratories, Peterborough, UK) (**Supplementary Figure S2**). Control experiments consisted of cells incubated in the same conditions but without sialidase enzyme or incubated with heat-inactivated sialidase (sialidase boiled for 20 min at 100ºC). After treatment, cell viability was evaluated by staining with 7-Aminoactinomycin D (7AAD, BD Biosciences) and annexin V and analyzed by flow cytometry (**Supplementary Figure S3**).

Proliferation Assays

T cell proliferation was accessed based on the carboxyfluorescein succinimidyl ester (CFSE) dilution assay. Briefly, T cells were labeled with CFSE, according to manufacturer instructions and then co-cultured with DCs in complete RPMI at 37 ºC. For experiments with

human cells, the MoDC: T cell ratio was 1:5 (0.4×10^5 DC: 2×10^5 T cells/well) and, 18h later, 10 IU/mL of IL-2 was added.

For experiments with murine cells, the sDC: OT-I or OT-II splenocytes ratio was 1: 2 (2.5×10^5 sDC: 5×10^5 T cells/well). The percentage of proliferated cells was calculated, by flow cytometric analysis, based on the percentage of population that showed reduced CFSE signal.

Antigen presentation assay

To evaluate antigen presentation in HLA-A*02:01 MoDCs, the cells were loaded with gp100 antigen peptides: $gp100_{280\text{-}288}$ short peptide (YLEPGPVTA) and $gp100_{280\text{-}304}$ long peptide (YLEPGPVTANRQLYPEWTEAQRLDC); that contain an amino acid sequence (underlined) that binds to the HLA-A*02:01 allele. MoDCs were incubated with several concentrations of gp100 peptides in RPMI medium, for 3 to 4 hours, at 37ºC. After three washing steps, MoDCs were co-cultured with gp100-specific $CD8^+$ T cells,[29] in IMDM medium, in a MoDC: T cell ratio of 3: 10. Following overnight incubation, the secretion of IFN-γ cytokine into supernatant was measured by Enzyme-linked immunosorbent assay (ELISA).

To assess the presentation of the OVA derived peptide SIINFEKL bound to $H\text{-}2K^b$ (murine MHC-I), sDCs loaded with OVA were stained with the anti-SIINFELK/$H\text{-}2K^b$ (25-D1.16 clone) mAb and analysed by flow cytometry.

Cytotoxicity Assays

$HLA\text{-}A^*02:01^+$ MoDCs loaded with tumor cell lysates were used to prime autologous $CD3^+$ T cells, as described above. Co-cultures were maintained for 2-3 weeks, and T cells were pulsed every week with $HLA\text{-}A^*02:01^+$ antigen-loaded MoDCs. IL-7 (5 ng/mL) was added at day 1 of culture and at the days of MoDC reload; and IL-2 (10UI/mL) was added at day two. Primed T cells were then cultured against target tumor cells MCF-7-GFP, in a ratio of 5 T cells per 1 tumor cell, for 5h at 37ºC. The apoptosis of MCF-7-GFP was determined based on the acidification of cell cytoplasm, which leads to decreased or complete loss of GFP fluorescence intensity. Thus, the percentages of tumor cells that do not undergo GFP fluorescence loss, based on flow cytometry analysis, were used to estimate the percentage of

cell survival. [30] The T cell cytolytic capacity, i.e., T cell degranulation, was evaluated by detecting at cell surface the lysosomal-associated membrane protein 1 (CD107a, LAMP-1), expressed in the membrane of cytotoxic granules.[31] Briefly, anti-CD107a mAb was added to the T cell: tumor cell co-cultures and after 1h, brefeldin A was added to block protein secretion. Following incubation for 4 h at 37ºC, the expression of the CD107a molecules was evaluated in $CD3^+$ cells, by flow cytometry.

For experiments with murine cells, splenocytes from OT-I and OT-II mice were primed by incubation with OVA-loaded sDCs, for 72h, as described above. The mouse melanoma cell line B16-OVA was labeled with CFSE, following the manufacturer instructions, and used as target tumor cell. The primed splenocytes were added to the B16-OVA cells at a ratio of 5 splenocytes per 1 tumor cell and co-cultured for 24h at 37°C. Tumor cell killing was evaluated by flow cytometry analysis, based on annexin-V and 7-ADD staining of $CFSE^+$ cells.

Cytokine analysis

The cytokine production was measured using ELISA technique, according to the manufacturer's instructions. Human IL-4, IL-6, IL-10, IL-12, TNF-α and IFN-γ ELISA development kits were purchased from Immunotools. Mouse IL-6, TNF-α and IFN-γ ELISA development kits were purchased from Prepotech (Hamburg, Germany). Cytokine concentration was calculated as pg/mL using the specific standard curves.

Statistical analysis

Data were analyzed using GraphPad Prism 6 (GraphPad Software, Inc). The significance of differences between series of results with three or more groups was assessed using the one-way ANOVA. Data with two groups were analyzed by Student-t-test. Tests were considered statistically significant when $p< 0.05$ (*), $p< 0.01$ (**) and $p< 0.005$ (***).

Results

Desialylated MoDCs loaded with whole tumor antigens have a higher ability to induce anti-tumor T cell responses

We first sought to assess the effect of sialic acid removal from the cell surface on the ability of DCs generated by methods employed in clinical trials to induce specific anti-tumor T cell responses. Therefore, we primed T cells with autologous desialylated MoDCs loaded with MCF-7-tumor cell lysates. Based on the CFSE dilution method, T cells primed with desialylated MoDCs showed a robustly higher proliferation, when compared to those primed with fully sialylated MoDCs (**Figure 1A**). To further characterize the profile of T cells generated by desialylated MoDCs exposure, the secretion of the Th1 cytokines - TNF-α and IFN-γ - and the Th2 cytokine - IL-4 - was analyzed. As shown in **Figure 1B,** when compared with T cells primed by fully sialylated MoDCs, T cells primed by desialylated MoDCs secreted slightly higher levels of IFN-γ and TNF-α. The expression of IL-4 by T cells was not altered when primed either by fully sialylated or desialylated MoDCs (**Figure 1B)**.

Thus, desialylated MoDCs have improved capacity to prime autologous T cells, as shown by their ability to induce T cell proliferation and production of Th1 cytokines, which are crucial for the induction of anti-tumor immune responses.

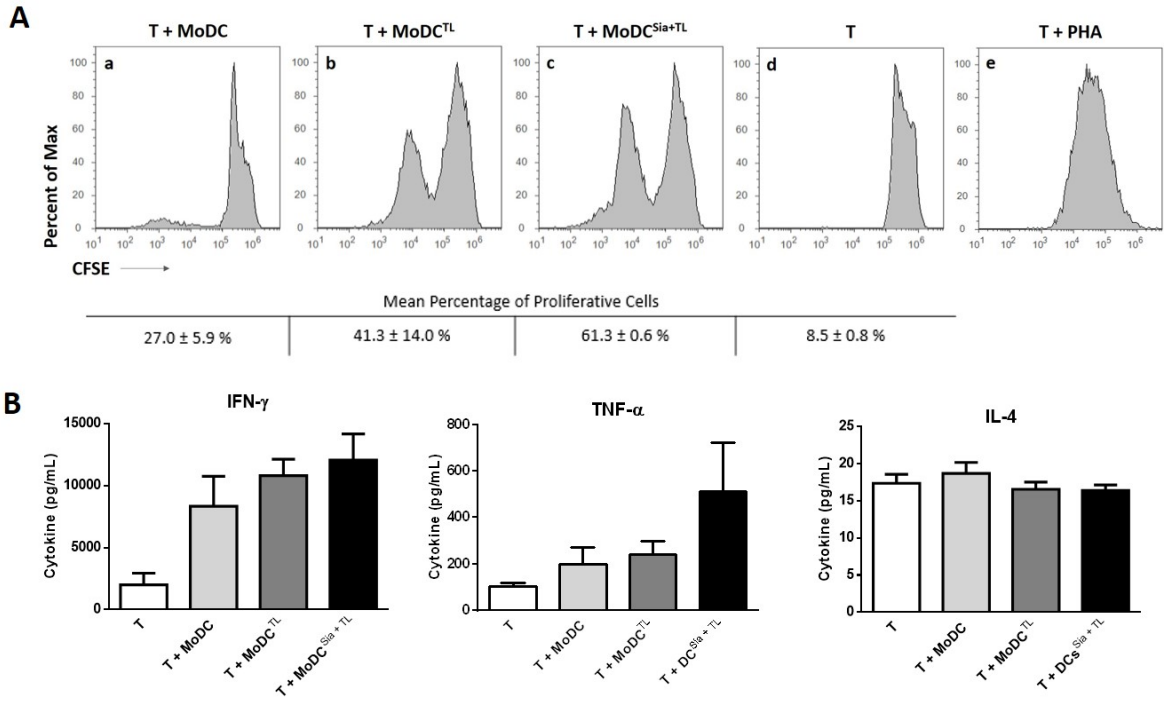

Figure 1. Desialylation of human MoDCs loaded with whole tumor cell antigens induces T cell activation. HLA-A*02:01$^+$ MoDCs were first treated with sialidase (Sia), for 1 hour at 37ºC or left untreated, followed by

loading with MCF-7 lysates (TL), as source of whole tumor cell antigens. MoDCs were then used in co-cultures with autologous T cells for 4-8 days in the presence of 10 IU/mL of IL-2. **(A)** *Induction of T cell proliferation by MoDCs*. T cells were previously labeled with CFSE and the progeny, i.e., the percentage of T cells that proliferated, was estimated by flow cytometry, based on the CFSE dilution. The histograms show representative experiments where T cells were co-cultured with: unloaded MoDCs (panel a), MoDCs loaded with MCF-7 lysates (panel b) and sialidase treated MoDCs loaded with MCF-7 lysates (panel c). Unstimulated T cells (panel d) served as negative control and phytohaemagglutinin (PHA)-stimulated T cells (panel e) as positive control. Tabled values represent the mean percentage of proliferative cells ± SEM of 2 independent assays. **(B)** *Th1 cytokines secreted in DC: T cell co-culture*. The cytokines secreted into the co-culture supernatants, following T cell stimulation with MoDC were measured by ELISA (n=6). Graph values represent the mean concentration (pg/mL) ± SEM.

The cytotoxicity of primed HLA-A*02:01^{+} T cells was then tested against MCF-7 breast cancer cells stably expressing GFP (MCF-7-GFP). The viability of the tumor cells was measured based on evaluation of loss of fluorescence due to cytoplasm acidification in the MCF-7-GFP cells. The degranulation of cytotoxic granules from T cells was also evaluated by the appearance of CD107a (LAMP-1) at T cells' surface. As shown in **Figure 2A,** tumor cell viability was significantly decreased when incubated with T cells that had been previously primed with desialylated MoDCs loaded with tumor-antigens, compared to those incubated with T cells primed with fully sialylated MoDCs. In agreement, the highest degranulation of T cells was observed when the cells were primed with desialylated MoDCs, as shown by the higher number of T cells expressing cell surface CD107a (**Figure 2B**).

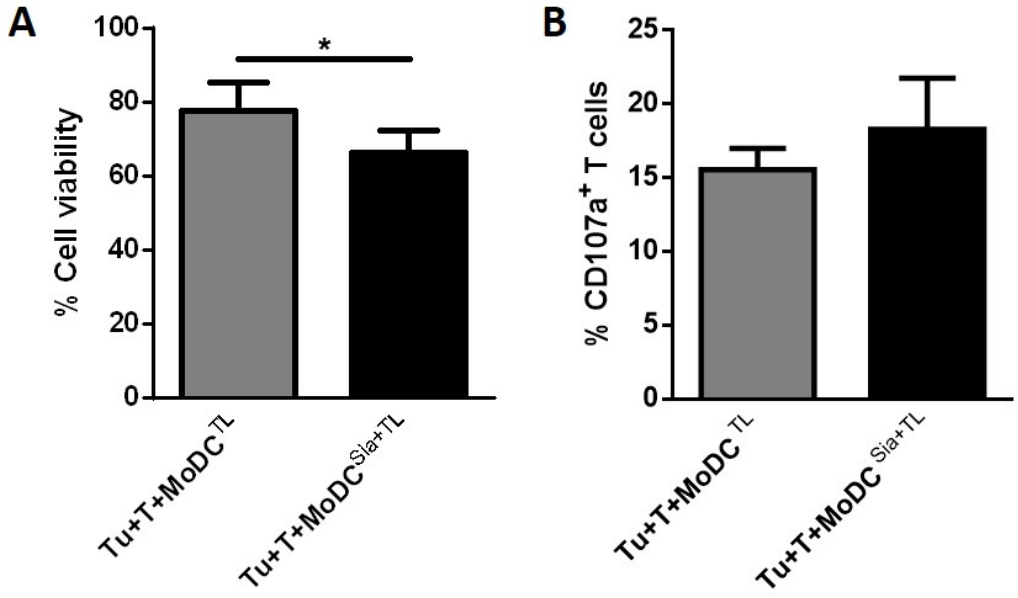

Figure 2. Desialylation of human MoDCs loaded with whole tumor cell antigens improves T cell cytotoxicity against tumor cells. MoDCs obtained from HLA-A*02:01 donors were treated with sialidase (Sia), for 1 hour at 37ºC or left untreated, followed by loading with lysates of the breast cancer cell line MCF-7 (TL). Autologous CD3^{+} T cells (T) were co-cultured with MoDCs loaded with MCF-7 lysates (MoDC^{TL}), or MoDCs that were sialidase treated and loaded with MCF-7 lysates (MoDC^{sia+TL}). After 3 weeks, CD3^{+} T cells were co-cultured for 5 hours with MCF-7 cells (Tu) in a ratio of 1 Tu: 10 T cells; non-stimulated T cells were

cultured with MCF-7 as control. (**A**) *T cells primed with desialylated human MoDCs loaded with MCF-7 lysates induce higher tumor cell death***.** Tumor cell apoptosis was evaluated by assessing the intensity of GFP reporter in MCF-7 tumor cells. Graph values represent the percentage ± SEM of viable tumor cells (GFP^+) of four independent assays. Statistically significant differences were calculated using t-test (*$P < 0.05$) **(B)** *T cells primed with desialylated MoDCs show higher degranulation.* The degranulation of cytotoxic T cells against tumor cells was determined by the percentage of T cells expressing CD107a at cell surface. Graph values represent the percentage ± SEM of $CD107a^+$ T cells of at least four independent assays.

Desialylated human MoDCs loaded with tumor antigens show higher levels of maturation and co-stimulatory markers and IL-12 secretion

To shed light on the mechanism behind the observed higher anti-tumor T cell responses induced by desialylation of MoDCs, we analyzed the maturation profile of desialylated MoDCs, loaded or not with tumor-antigens. For that, the expression of maturation markers, namely the MHC-I and MHC-II antigen-presenting molecules, and the expression of co-stimulatory molecules, such as CD80 and CD86, were assessed after sialidase treatment. Sialidase treatment of unloaded MoDCs significantly increased the expression of all maturation markers compared to untreated MoDCs (**Figure 3A, left panels**). Similarly, a significantly improved maturation phenotype was observed after sialidase treatment of MoDCs loaded with MCF-7 lysates (**Figure 3A, right panels**); yet, sialidase effect was restricted to MHC-I and MHC-II molecules, and did not extent to the co-stimulatory molecules CD80 and CD86 (**Figure 3A, right panel**). The combination of sialidase treatment with tumor-antigen upload did not alter the expression of other co-stimulatory molecules, such as CD40, and of the chemokine receptor CCR7, when compared with fully sialylated MoDCs (results not shown). Given the Th1 profile of T cells primed by desialylated MoDCs, we further evaluated the effect of sialic acid removal from DC surface on their ability to secrete Th1-inducing cytokines, IL-12, TNF-α and IL-6, and the immune-inhibitory Th2-inducing cytokine IL-10. A significant increase in the expression of IL-12 was observed in desialylated MoDCs loaded with tumor antigens compared with those that were not treated with sialidase (**Figure 3B**).

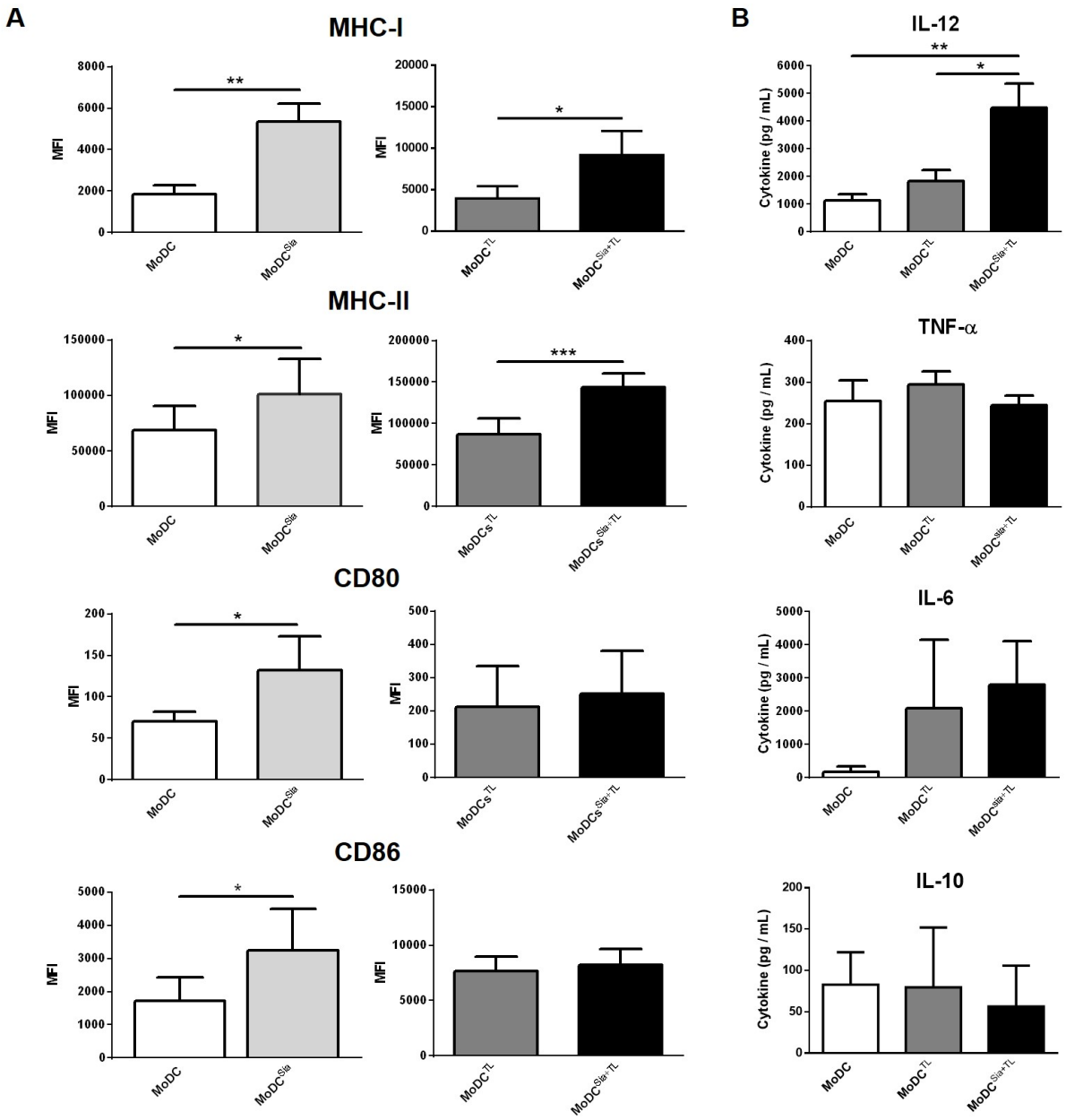

Figure 3. Desialylated MoDCs show higher levels of maturation and expression of pro-inflammatory cytokines (**A**). *Desialylated MoDCs show a higher maturation phenotype than fully sialylated MoDCs.* The expression of several maturation markers was evaluated by flow cytometry. MoDCs were first treated with sialidase (Sia) for 1h at 37ºC and then loaded (A, right panel) or not (A, left panel) with MCF-7 lysates (TL), for 3h at 37ºC, as described in Materials and methods. Graph values represent the mean fluorescence intensity (MFI) (average±SEM) of at least three independent assays. Statistically significant differences were calculated using t-test (*$P < 0.05$, **P < 0.01 and ***P < 0.0001) and refers to the difference between untreated and sialidase treated conditions. (**B**) *Sialidase treatment of MoDCs elicits IL-12 secretion.* The cytokines secreted by MoDCs following sialidase treatment were measured by ELISA. Data represent the cytokine concentration (pg/mL average±SEM) of 6 independent experiments. Statistically significant differences were calculated using t-test (*$P < 0.05$ and **P < 0.01) and refers to the difference between untreated and sialidase treated conditions.

Our data show that desialylation of MoDCs boosts DC maturation, in particular the expression of MHC-I and MHC-II, and secretion of the Th1-inducing cytokine IL-12.

Desialylated murine splenic DCs show higher levels of maturation and co-stimulatory markers

To investigate whether the sialidase effect was restricted to human DCs, we analysed the maturation profile of desialylated murine sDCs. Therefore, we analysed the expression of MHC and co-stimulatory molecules by sDCs after sialidase treatment. sDCs that were treated with sialidase showed a significantly higher expression of H-2k^b (MHC-I), IA^b (MHC-II), CD80 and CD86, molecules (**Figure 4**). Thus, desialylation of murine sDCs leads to a stronger induction of maturation, similar to human MoDCs.

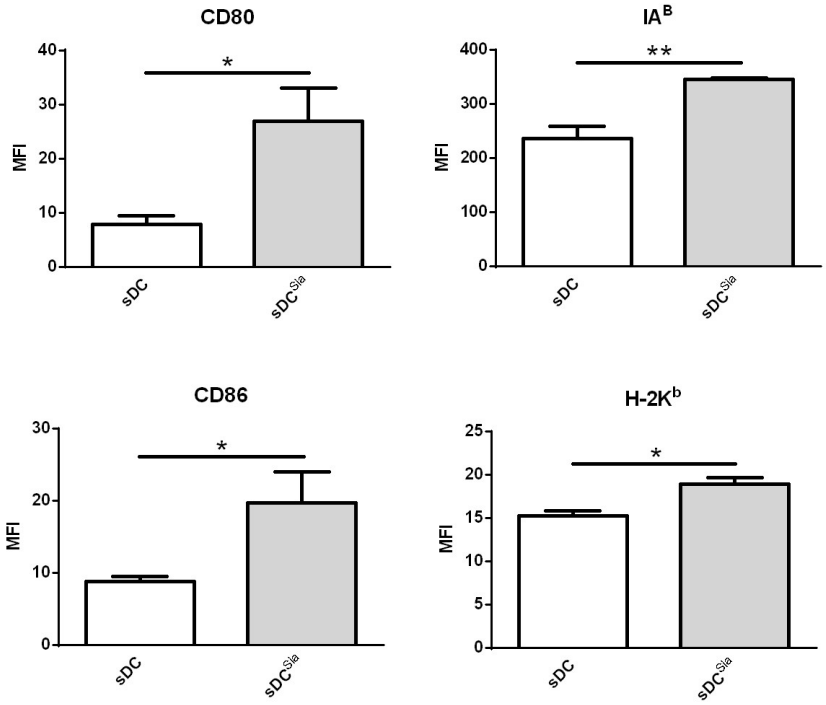

Figure 4. Sialidase treatment of murine sDCs induces their maturation. *Evaluation of maturation markers after sialidase treatment*. Murine sDCs were treated with sialidase or left untreated and the maturation markers evaluated by flow cytometry. Statistical significance (*P < 0.05 or **P < 0.001) refers to the difference between fully sialylated and desialylated sDCs, calculated using t-test. Values represent the means of the MFI of at least six independent assays.

Desialylation of murine splenic DCs induces antigen-specific CD4$^+$ and CD8$^+$ T cell proliferation and activation

Since desialylated sDCs showed an increased maturation phenotype, we next examined whether desialylation would result in a better ability of sDCs to prime antigen specific T cells. To assess this, sDCs were loaded with the model antigen OVA, desialylated and then co-cultured with either OVA-specific CD4$^+$ or CD8$^+$ T cells, from transgenic OT-II or OT-I mice, respectively. T cell proliferation, expression of activation markers and cytokine

secretion by T cells, were assessed. Compared with fully sialylated OVA-pulsed sDCs, desialylated OVA-pulsed sDC induced significantly higher OVA-specific $CD4^+$ T cell proliferation (**Figure 5A**), as determined by CFSE dilution method. Accordingly, the activation of $CD4^+$ T cells was significantly improved when these cells were primed with desialylated OVA-pulsed sDCs, as demonstrated by the evaluation of the percentage of $CD69^+CD44^{high}$ T cells. To assess whether priming with desialylated sDCs would induce a Th1 profile on $CD4^+$ T cells, we analyzed the levels of IL-6, TNF-α and IFN-γ secretion by primed $CD4^+$ T cells. As shown in **Figure 5A,** the secretion of all three Th1-cytokines was significantly up-regulated when $CD4^+$T cells were primed with desialylated OVA-pulsed sDCs compared to T cells primed with fully sialylated OVA-pulsed sDCs. Taken together, these results show that sialidase treatment of sDCs significantly improves their ability to induce the proliferation and activation of $CD4^+$ T cells with improved Th1 profile.

Regarding $CD8^+$ T cells, desialylated sDCs showed a modest tendency to induce proliferation, but a considerable ability to activate them, based on the percentage of $CD69^+CD44^{high}$ T cells (**Figure 5B**). The secretion of IL-6, TNF-α and IFN-γ cytokines were not significantly altered when $CD8^+$ T cells were primed by desialylated sDCs (**Figure 5B**).

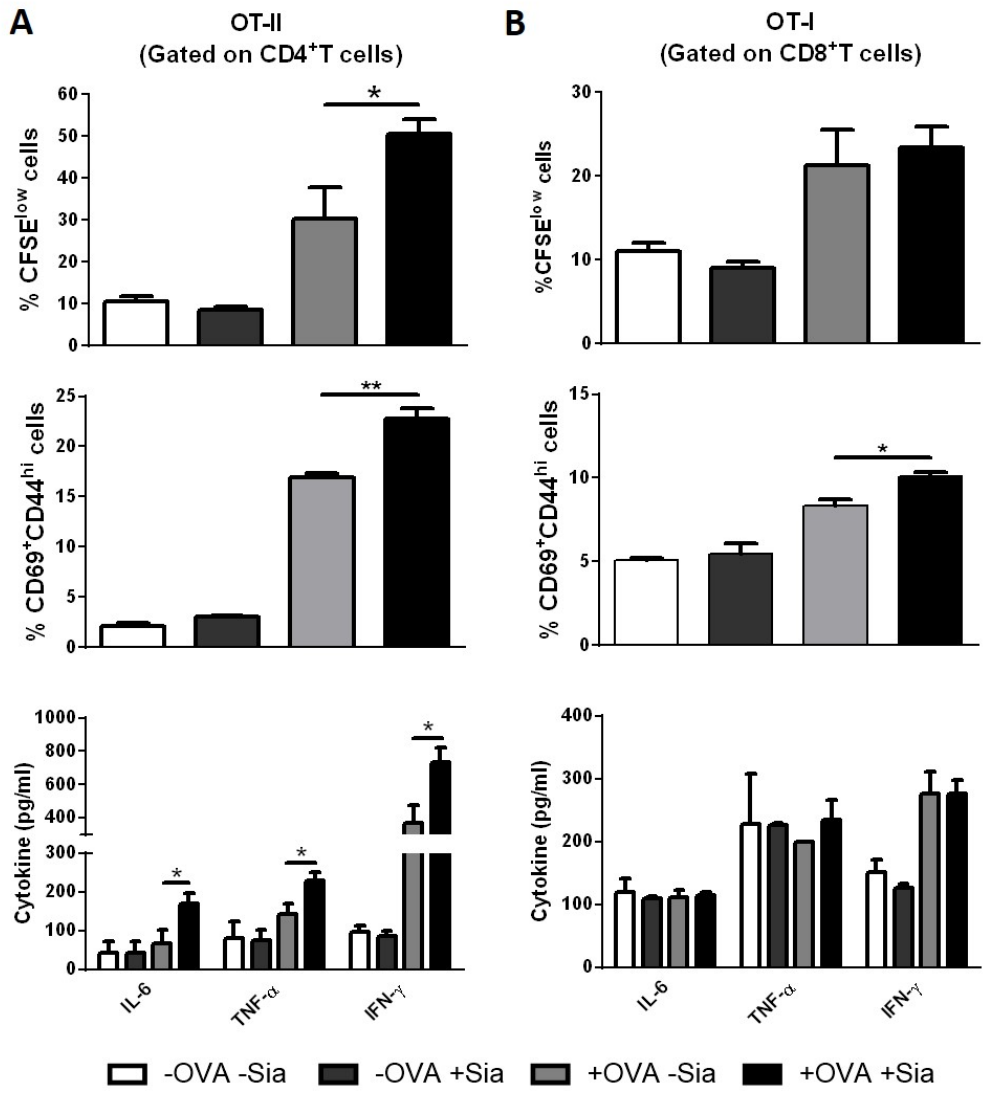

Figure 5. Sialidase treatment of murine sDCs induces OVA-specific $CD4^+$ and $CD8^+$T proliferation and activation. Murine **s**DCs were pulsed with OVA for 4h and treated or not with sialidase for 1h. **A)** *Desialyated sDCs strongly induce proliferation, activation and differentiation of $CD4^+$T into a Th1 effector phenotype.* Splenocytes from OT-II were isolated and co-cultured with the sDCs for 72h in a ration of 1:2 (DCs: splenocytes). After 72 h, the percentage of proliferating and activated $CD4^+$T cells was determined using a CFSE dilution and evaluating of surface expression CD69 and CD44, respectively (gating on $CD4^+$ T cells). Flow cytometry analysis show an increase in proliferation of $CD4^+$T cells primed with fully sialylated OVA-pulsed DC. We assessed for IFN- γ, IL-6, and TNF-α secretion in the supernatants of co-cultures by ELISA; a significant increase in all cytokines tested was observed after $CD4^+$ T cells were co-cultured with desialylayed OVA-pulsed sDCs compared with fully sialylated ones. Statistical significance (*P < 0.05 or **P < 0.001), calculated using t-test, refers to the difference between fully sialylated and desialylated sDCs. B) *Desialyated sDCs induce activation of $CD8^+$T.* Splenocytes from OT-I were isolated and co-cultured with sDCs for 72h in a ration of 1:2 (DCs: splenocytes) The proliferation and activation of $CD8^+$T cells was determined by CFSE dilution and cell surface expression of CD69 and CD44 markers (gating on $CD8^+$ T cell). $CD8^+$ T cells that were treated with sialidase-treated DCs showed a significant expression of CD69 and CD44 markers compared to fully sialylated OVA-pulsed sDCs (*P < 0.05). The levels of IFN- γ, IL-6, and TNF-α production in the supernatants of co-cultures were evaluated by ELISA; no significant changes were observed.

Desialylation of murine splenic DCs leads to improved T cell-mediated tumor cell death

To investigate whether OVA-specific $CD4^+$ and $CD8^+$ T cells primed with desialylated sDCs would have a higher ability to induce tumor cell cytotoxicity, we co-cultured the primed T cells with the OVA-expressing melanoma cell line, B16-OVA. Interestingly, B16-OVA tumor cells that were incubated with either $CD4^+$ (**Figure 6A**) or $CD8^+$ T cells (**Figure 6B**), previously primed with desialylated OVA-pulsed sDCs, showed significantly less viability than those incubated with T cells primed with fully sialylated OVA-pulsed sDCs (**Figure 6**). Thus, desialylation of sDCs improves their ability to prime antigen-specific T cell-mediated immune response against tumor cells.

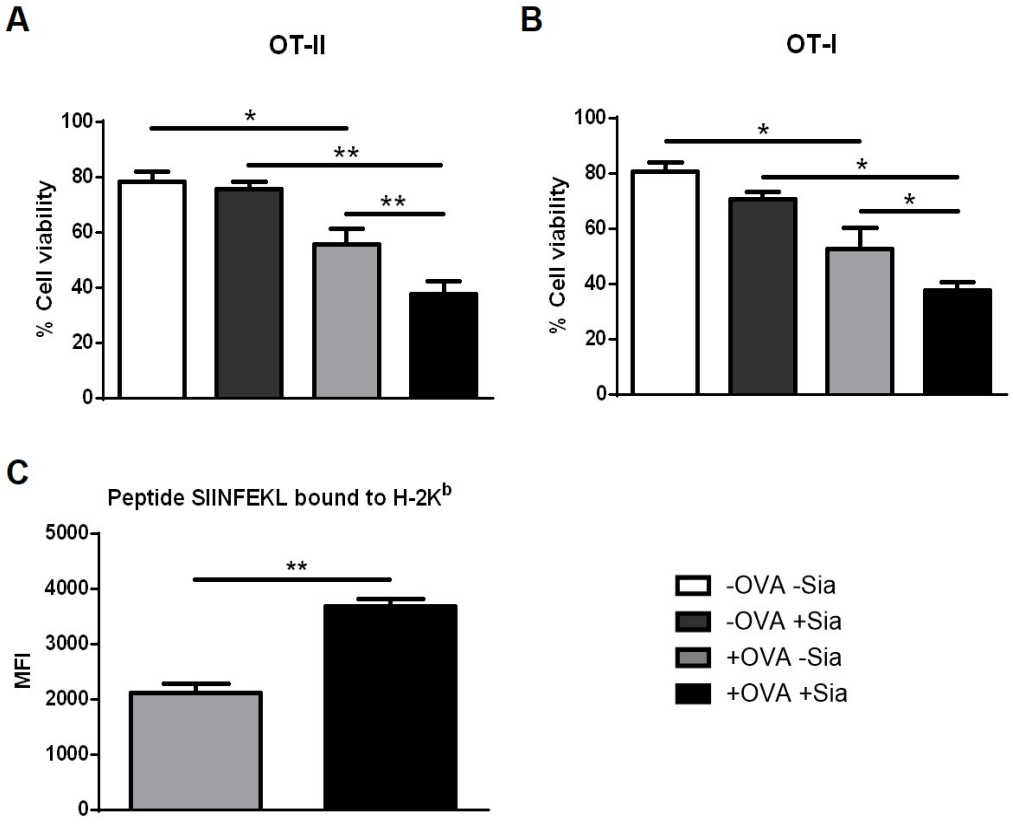

Figure 6. Sialidase treatment of murine sDCs loaded with OVA improves T cell cytotoxicity against tumor cells. Induction of cytolysis of tumor targets was tested by co-culturing untreated (light grey bars) or sialidase treated (black bars) OVA-pulsed DCs and splenocytes from either OT-II (A) or OT-I (B) mice for 72h. After, primed T cells and OVA-expressing B16 mouse melanoma cells (B16-OVA) were co-cultured for 24h. B16-OVA tumor cell death was assessed by staining with 7-AAD and annexin-V and analyzed by flow cytometry. Sialidase treatment of OVA-pulsed sDCs induced a higher tumor cell cytotoxicity compared to fully sialylated ones. Statistical significance (*$P < 0.05$ or **$P < 0.001$) refers to the difference between fully sialylated and desialylated sDCs (n=2). C) *Desialylated sDCs show improved presentation of endocytosed ovalbumin-derived peptide SIINFEKL, bound to MHC class I.* Murine splenic DCs were first incubated with OVA and treated (black bars) or not (grey bars) with sialidase. After these treatments, the presentation of the ovalbumin-derived peptide SIINFEKL bound to H-2K^b (murine MHC class I) epitopes was analysed by flow cytometry, using the 25-D1.16 monoclonal mAb. Graphs show the MFI ± SEM of two independent assays. Statistical significance

(**P < 0.001) refers to the difference between fully sialylated and desialylated sDC and it was determined using t-test.

Desialylated murine sDCs showed improved presentation of OVA-derived peptide

The presentation of antigens through MHC-I, by DCs to cytotoxic T cells, includes the cross-presentation of antigens, an essential mechanism to present endocytosed exogenous antigens via MHC-I.[9]

Since the activation of $CD8^+$ T cells by OVA-pulsed sDCs implies cross-presentation of peptides derived from the endocytosed OVA, via MHC-I, we then assessed the ability of sDCs to cross-present OVA-derived peptides through MHC-I. For this purpose, after incubation with OVA, and treatment with sialidase, sDCs were stained with the 25-D1.16 mAb that specifically recognizes the processed OVA peptide- SIINFEKL bound to H2-K^b. This assay readily detected cross-presenting murine sDCs and showed that, sialidase treated sDCs had a significant higher cross-presentation of the SIINFEKL peptide through MHC-I (**Figure 6C)**. Thus, the data indicates that desialylation of murine sDCs improves the cross-presentation of antigens, via MHC-I.

Sialidase treatment improves antigen presentation via MHC-I by human MoDCs

We next determined whether improved antigen cross-presentation was also extensible to human desialylated DCs. Additionally, we also analyzed whether DC desialylation would affect the presentation of exogenously supplied high affinity peptides that bind directly to MHC-I molecules, without previous cell internalization.

To address this, we loaded HLA-A*02:01^+ MoDCs with short and long peptides derived from the gp100 tumor antigen. The peptides share the $gp100_{280–288}$ sequence (YLEPGPVTA) that binds to HLA-A*02:01. The short peptide binds directly to MHC-I, whereas the long peptide needs to be internalized, conducted into phagosomes and processed to be incorporated into MHC-I, before it is cross-presented. The MoDCs were then loaded with both peptides and their ability to prime $gp100_{280–288}$ specific $CD8^+$ T cell clones was evaluated, based on their IFN-γ secretion ability. As shown in **Figure 7**, desialylated MoDCs loaded either with gp100 short (**Figure 7A**) or with long peptide (**Figure 7B**) showed higher ability to stimulate

$CD8^+$ T cells. The data suggests that desialylation of MoDCs improves antigen presentation through MHC-I. This is due to an increase in antigen cross-presentation and to the fact that exogenously supplied peptides bind to desialylated MHC-I with higher affinity.

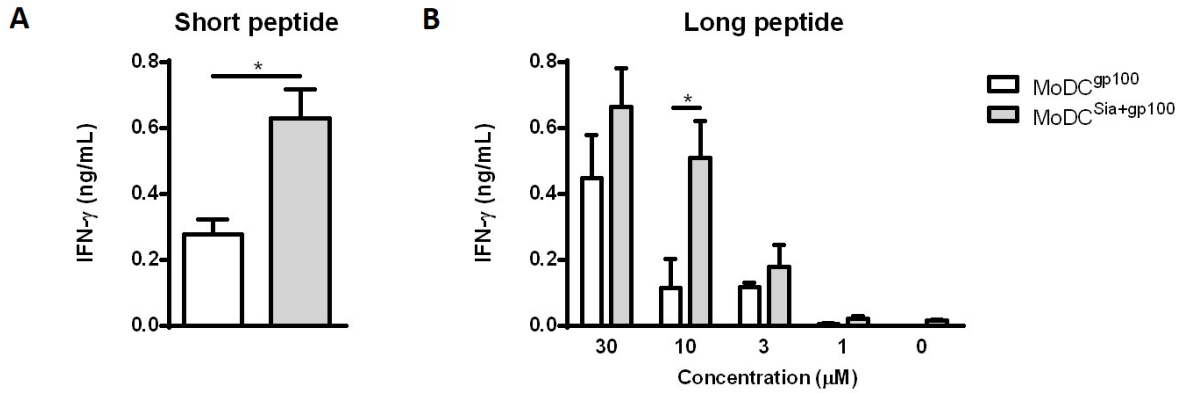

Figure 7. Sialidase treatment of human MoDCs improves antigen cross-presentation via MHC-I to $CD8^+$ T cells. Gp100-specific $CD8^+$ T cells were co-cultured overnight with gp100-loaded MoDCs, that have been previously treated or not with sialidase ($MoDC^{sia+gp100}$ and $MoDC^{gp100}$, respectively). Short gp100 peptides (**A**) and different concentrations of the long gp100 peptide (**B**) were used. To evaluate the activation of $CD8^+$ T cells, the secretion of IFN-γ cytokine was measured by ELISA (mean± SEM) (n= 3). Statistical significance (*$P < 0.05$) refers to the difference between fully sialylated and desialylated gp100-loaded MoDCs.

Discussion

The use of DC-based vaccines for active immunotherapy of cancer patients has gained increasing interest among scientific community.[32] However, the successful use of these cells, as a cancer vaccine, has been limited and only a small percentage of objective clinical responses has been observed in clinical trials.[4,33] The immediate hurdle for the success of DCs immunotherapy is to improve their ability to prime T cells, so they can elicit an effective and long-lasting anti-tumor immune response.

Terminal sialylation of cell surface glycans is involved in several immune physiological mechanisms, including immune recognition and regulation of immune-cell differentiation and maturation. We were the first to report that human MoDCs, which are commonly employed in clinical trials (e.g., NCT00834002, NCT00814892, and NCT01525017) display a high content of α(2,6)- and α(2,3)- sialic acid linked to cell surface glycoconjugates[24] and that

maturation of MoDCs decreases cell surface sialylation, due to a down-regulation of sialyltransferase activity.[25,34] Also, sialic acid removal or deficiency improves the DCs' maturation status.[25,35] On the other hand, MoDCs with a higher α(2,6)-linked sialic acid content exhibit a more tolerogenic and immature phenotype,[36] suggesting that sialylation of MoDCs may be involved in immune-dampening. Yet, it is unknown whether shortage of cell surface sialic acid level affects the ability of MoDCs to induce proper anti-tumor immune responses. Accordingly, in this study we assessed the ability of desialylated MoDCs to induce anti-tumor immune responses. To address this, desialylated MoDCs were generated using a recombinant sialidase, which rapidly leads to a transient cell surface desialylation, while preserving MoDC viability. Desialylation of MoDCs was observed up to 24h, after enzymatic treatment and then, after 48h, MoDC have already recovered their typical sialic acid content (data not shown), probably due to protein turnover.

Human desialylated MoDCs, pulsed with whole tumor antigens from the breast cancer cell line MCF-7 induced a significant higher proliferation of T cells, compared to fully sialylated MoDCs. Increased T cell proliferation was accompanied by an increased secretion of IFN-γ and TNF-α, typical of a Th1-induced response. Concomitantly, T cells primed by desialylated MoDCs pulsed with tumor cell lysates exhibited the highest cytotoxic activity against MCF-7 tumor cells. This higher capacity of desialylated antigen-loaded MoDCs to prime T cells may be due to the fact that desialylated MoDCs show an increased expression of antigen-presenting and co-stimulatory molecules, which is in agreement with our previous reports.[25] Furthermore, desialylation of MoDCs loaded with tumor cell lysates resulted in a remarkable higher secretion of IL-12 cytokine. This cytokine triggers the differentiation of $CD4^+$ T cells into IFN-γ producer's cells, thus inducing Th1 responses. The secretion of IL-10 was not significantly affected but showed a slight reduction in desialylated MoDCs. This is in agreement with the induction of a Th1 profile, since IL-10 blocks differentiation of *naïve* T cells into Th1 effector cells[37,38] and limits maturation of DCs by down-regulating co-stimulatory molecules and IL-12 production.[39,40] Overall, sialidase treatment of human MoDCs loaded with tumor antigens induces an $IL\text{-}12^{high}$ $IL\text{-}10^{low}$ DC phenotype, that promotes DCs maturation and T cell differentiation into Th1 effectors, crucial for the generation of anti-tumor responses. Though, increased maturation of desialylated MoDCs had been described previously, these results are the first to indicate that desialylated MoDCs loaded with tumor antigens have higher capacity to prime T cell-mediated anti-tumor immune responses and that this is probably due to an increased maturation state.

We then determined whether the desialylation effect was extensible to murine DCs. By using murine sDCs loaded with the model antigen OVA, we assessed their capacity to prime antigen specific $CD4^+$ and $CD8^+$ T cells against OVA-expressing cancer cells. The results obtained with this model are the first showing that desialylation also induces maturation of murine sDCs, by improving the expression of the antigen presenting molecules MHC-I and -II and the co-stimulatory molecules CD80 and CD86. Moreover, desialylated sDCs, pulsed with OVA antigens showed a remarkable higher ability to boost proliferation and activation of OVA-specific $CD4^+$ T cells, and secretion of IFN-γ, IL-6 and TNF-α. Notably, both $CD4^+$ and $CD8^+$ OVA-specific T cells primed by desialylated OVA-pulsed DCs, exhibited higher cytotoxic activity towards OVA-expressing tumor cells, when compared to those primed with fully sialylated OVA-pulsed sDCs.

Importantly, our data shows that sialidase treatment of DCs improves antigen cross-presentation in both murine and human DCs. Sialidase treatment of murine sDCs improved presentation of endocytosed ovalbumin-derived peptide SIINFEKL, bound to MHC-I, as assessed by increased reactivity with the 25-D1.16 mAb. Treatment of human HLA-A*02:01 MoDCs pulsed with synthetic long or short peptides from the tumor antigen gp100, displayed enhanced antigen presentation through MHC-I, as evaluated based on the increase of IFN-γ production by $gp100_{280–288}$ specific $CD8^+$ cytotoxic T cells. Since the long peptide needs to be internalized by MoDC, exported from lysossome to cytosol and then conducted into the cross-presentation process, our data is suggestive that desialylation improves antigen cross-presentation, which is in agreement with data obtained with murine sDCs. It is possible that desialylation affects critical cell surface receptors, that favor antigen export from lysosomes to cytosol, such as the mannose receptor[10], thus improving the mechanism of export of endocytosed antigens to the cytosol. Yet, data obtained with the short gp100 peptide that binds exogenously and directly to MHC-I, suggests that desialylation also improves binding of exogenous peptides to cell surface MHC-I. Noteworthy, our data also suggests that increased expression of MHC-I (either due to recycling, or newly synthesis) is a possible mechanism behind improved antigen presentation through MHC-I. Thus, future studies are envisaged to better understand how sialidase treatment of DCs induces superior antigen presentation to cytotoxic T cells.

Collectively, our data show that sialidase treatment of DCs is able to induce all the phenotypical and functional features of maturation, including increased antigen presentation, co-stimulatory molecules and secretion of Th1 cytokines. More important, this is the first

report showing that an enzymatic treatment through extrinsic desialylation of *in vitro* generated DCs elicits better presentation of tumor antigens through MHC-I, which can in turn activates anti-tumor immune responses. Interestingly, a previous study reported that desialylation of $CD8^+$ T cells is required and adequate for their effective anti-tumor response against glioma cancer.[41] In agreement, we have shown here that removal of sialic acid from cell surface of an immune cell considerably boosts its anti-tumor immune response. Therefore, our findings suggest that sialidase treatment of DCs can be used as a powerful tool to improve the effectiveness of clinical DC-based therapeutic cancer vaccines.

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Authorship Contributions and Disclosure of Conflicts of Interest

The study was conceived by P.V., who designed and supervised all research, funded the research (together with C.E.T.), and wrote the manuscript. M.S., Z.S and G.M. designed and performed research, collected and analyzed data, and wrote the manuscript. All other authors performed research, collected and analyzed data. All authors declare no competing financial interests.

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Supplementary Figures

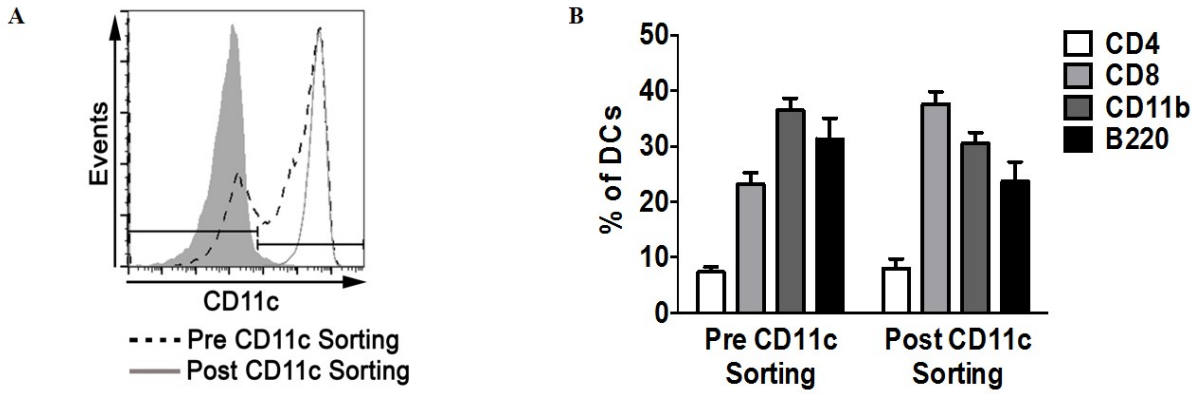

Figure S1. Characterization of splenic DC subsets after CD11c positive selection. (A) *Representative histogram of CD11c cells after positive selection*. After sorting, the purity of the population was typically around 97%. **(B)** Proportion *of each conventional sDC subset before and after CD11c cell sorting*. In order to assess whether CD11c positive selection would affect the distribution of the different subsets of splenic DCs, we stained CD11c cells with CD4, CD8a (lymphoid DCs), B220 (plasmacytoid DCs) and CD11b (myeloid DCs) markers. After CD11c positive selection, the relative proportions of different sDC subsets remained roughly similar, except for the lymphoid and plasmacytoid populations that respectively increased and decreased slightly.

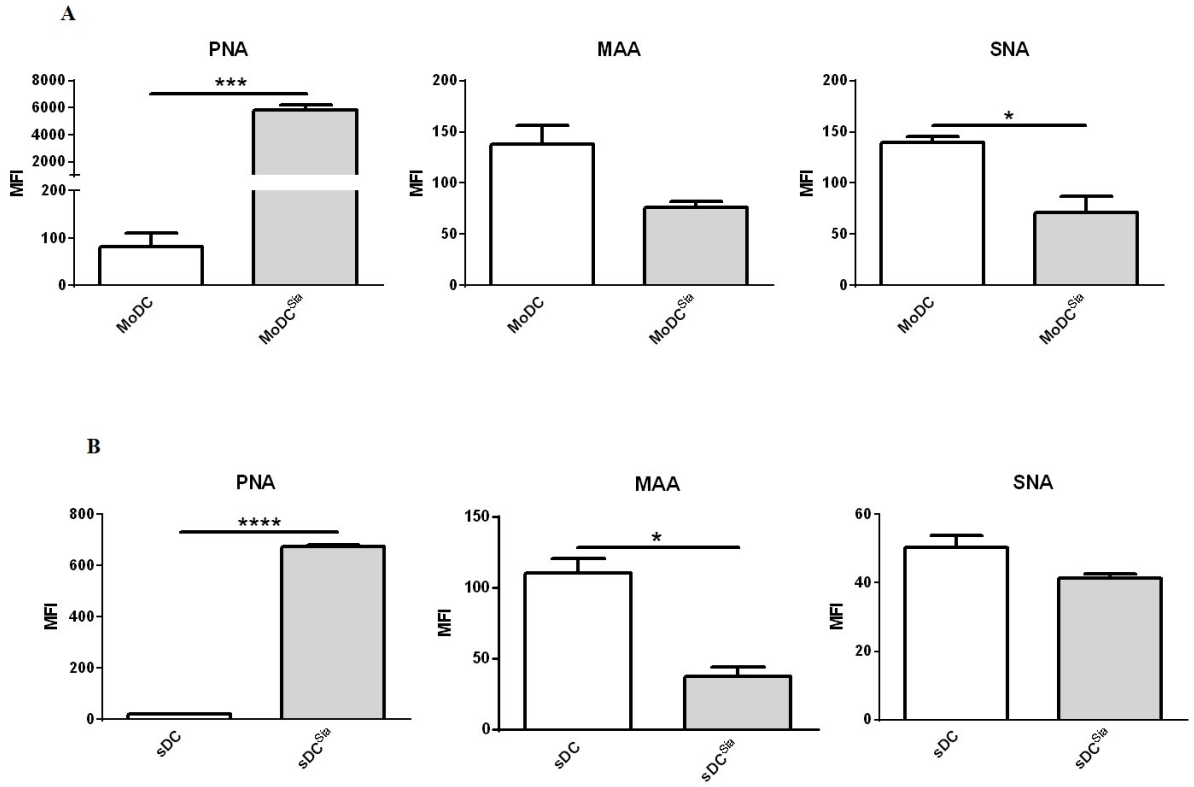

Figure S2. Lectin staining to test the efficacy of sialidase treatment. Human MoDCs (**A**) and murine sDCs (**B**) were treated with sialidase (grey bars) or left untreated (white bars) and stained with *Sambucus nigra lectin* (SNA; recognizing α(2,6)-sialic acids), *Maackia amurensis lectin* II (MAA; recognizing α(2,3)-sialic acids) and *Peanut agglutinin lectin* (PNA; recognizing T antigen- Galβ1-3GalNAcα1-Ser/Thr) and analyzed by flow cytometry. Values represent the means of the MFI of at least six independent assays. Statistical significance (*P < 0.05 or ***P < 0.0001) refers to the difference between untreated and sialidase-treated DCs. Sialidase treatment decreased MAA binding and increased PNA staining of both human MoDCs and murine sDCs, resulting from the removal of α(2,3)-linked sialic acids; removal of α(2,6)-linked sialic acids after sialidase treatment was detected by SNA staining decrease.

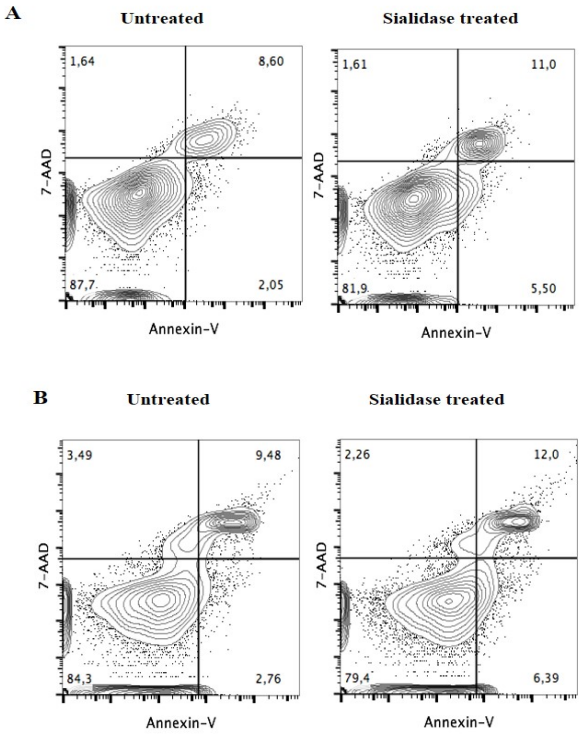

Figure S3. Assessment of cell viability after sialidase treatment of DCs. Human MoDCs (**A**) and murine sDCs (**B**) were treated with sialidase or left untreated and dual stained with Annexin V and 7-AAD. Both human and murine DCs treated with sialidase exhibit no significant increase in cell death compared with untreated ones, suggesting that DCs can tolerate sialic acid removal relatively well and remain viable to exert their immunologic function. Importantly, sialidase treatment of DCs did not compromise DCs viability.

Chapter 7

General discussion and Final conclusions

7. General discussion and Final conclusions

Glycosylation is the most common posttranslational modification on the cell surface and is involved in many physiological and pathological processes. Addition of glycan structures to protein or lipid scaffolds can modulate their biochemical and biophysical properties and therefore their functionality.[1] Specific carbohydrate determinants are associated with certain stages of cell differentiation and can modulate processes like cell-signaling and migration, host-pathogen interactions and tumor invasion and metastasis.[2,3] Specifically, several immune-related processes are intrinsically linked to certain glycosylation patterns. Specific innate and adaptive immune mechanisms critically depend on appropriate molecular and cellular glycosylation.[4]

One of the vital proprieties of the immune system is the ability to direct the accumulation of immune cellular effectors in a site-specific fashion so that a successful immune response can be settled.[5] In the context of an inflammatory response, migration of leukocytes to sites of tissue injury or infection crucially dependents on transient adhesive interactions between the vascular E-selectin expressed on endothelial cells and its carbohydrates counter-receptors expressed on cell surface of leukocytes.[6,7]

7.1 Comprehensive Analysis of Glycoprotein E-selectin Ligands Expressed on Human Peripheral Blood Mononuclear Cells

The body's immune system is specialized to respond rapidly to invading pathogens, a process which depends on constant surveillance and swift deployment of leukocytes at relevant tissues. Myeloid cells constitute one of the body's first lines of defense and are rapidly recruited at sites of injury.[8] Subsequently, an adaptive immune response may be mounted where naïve lymphocytes, which continually recirculate between the bloodstream and secondary lymphoid tissues in search of cognate antigen, may differentiate into effector lymphocytes that are able to migrate into inflammatory sites in peripheral non-lymphoid tissues.[8,9]

In **chapter 3** we performed a detailed and complete description of the E-selectin ligands expressed by the major subsets of peripheral blood mononuclear cells (PBMCs): monocytes, $CD4^+$ and $CD8^+$T-cells and B cells. Our data demonstrate that the ability of leukocytes to

bind to E-selectin under hemodynamic shear conditions is integrally correlated to the overall amount of E-selectin ligand determinants expressed by these cells. Interestingly, monocytes possess the highest ability to bind to E-selectin under hemodynamic shear stress and consistently showed the highest expression of sLe^X–bearing glycoproteins among PBMC subsets. Our data show, for the first time, that in addition to the cutaneous lymphocyte antigen (CLA)[10], monocytes also express non-CLA glycoproteins with E-selectin reactivity. Thus, we described here three new unreported monocytic E-selectin binding glycoproteins:(1) The glycoform of CD44 known as Hematopoietic cell E-/L selectin ligand (HCELL); (2) The glycoform of CD43, CD43E; and (3) An unidentified ~70 kDa glycoprotein. After monocytes, $CD4^+$T cells are the leukocytes that exhibit the highest ability to bind to E-selectin under shear stress conditions and accordingly, express higher amounts of E-selectin binding determinants than $CD8^+$T or B cells. $CD4^+$T cells express the previously reported E-selectin binding glycoforms of PSGL-1 (i.e., CLA) and CD43 (i.e., CD43E),[11,12] and, remarkably, we observed that these cells also express HCELL. On the contrary, peripheral $CD8^+$T and B cells express low to no levels of E-seletin ligands and therefore show little ability to bind to E-selectin under blood flow conditions. $CD8^+$T cells express lower amounts of CLA and CD43E compared to $CD4^+$T cells and do not express HCELL, whereas B-cells completely lack glycoproteins with E-selectin ligand activity.

The differential E-selectin ligand expression profiles among PBMCs may elucidate, at least in part, their distinct kinetics of migration into inflamed sites during an acute inflammatory response. Leukocyte recruitment to sites of injury has been always assumed to result mainly from leukocyte chemotaxis, i.e., the ability of specific leukocyte subsets to respond to secreted small molecule chemical factors that would enable their accumulation to injured tissue.[13-16] However, our data suggest that the leukocyte E-selectin binding activity may play the principal role in the kinetics of recruitment of the different PBMCs into sites of inflammation.

Upon tissue wounding, monocytes are among the first innate immune cells to arrive to the injured site.[9] Indeed, adherence of circulating monocytes to endothelial monolayer and subsequent transmigration and accumulation of these leukocytes at the injured site represent one of the most important events in the initiation of the inflammatory process.[9,17] Then, as the innate immune system yields to adaptive immunity in the elaboration of immunoreactivity, subsequent migration of lymphocytes occurs within days. Hence, after antigen presentation via APCs in the central lymphoid organs, *naïve* T cells become activated and differentiate

into effector or memory T cells, which leave the lymphoid organs and migrate to inflamed peripheral sites, through blood circulation. Although peripheral homing properties by $CD4^+$ and $CD8^+$T cells are still in debate, several studies reported that $CD4^+$T cells arrive and accumulate at the injured tissues before $CD8^+$T cells.[18-20] Indeed, in some peripheral tissues, such as vaginal tissue and skin, the presence of $CD4^+$T cells seems to be critical for the recruitment and entry of activated $CD8^+$T cells.[18,21] Regarding B cells, their migration into peripheral tissue from bloodstream is poorly understood. In contrast to T cells, B cells do not migrate into normal peripheral tissues.[19] In inflammatory milieu, various *in vivo* studies reported a poor ability of B cells to migrate into sites of acute inflammation,[22,23] while others reported that these cells can accumulate in a very localized manner into infected tissue in acute or chronic inflammatory conditions, although the tempo of B cells migration into the inflamed tissue is much lower compared to T cells.[19,24]

Notably, our results are in agreement with the kinetics of PBMC migration into inflamed sites, previously described in the literature. Therefore, a parallelism may be traced between the levels of leukocyte E-selectin ligand expression and their ability to migrate and accumulate into inflamed sites. Particularly, the wide repertoire of glycoprotein E-selectin ligands expressed by monocytes, including the highly potent HCELL[25], would give them the advantage to accumulate to injured sites within a few hours after infection/wound challenge. By contrast, the poor ability of B cells to migrate into inflamed tissue[23] can be due to their incapacity to bind to E-selectin.

To gain further molecular understanding of the scarcity of E-selectin ligand expression in B cells, we performed quantitative PCR studies of relevant glycosyltransferases directing sLe^X synthesis on B cells and monocytes. Strikingly, our results showed that peripheral blood B cells have low gene expression of the glycosyltransferases involved in the biosynthesis of sLe^X, namely $\alpha(1,3)$-fucosyltransferases, and high expression of competing glycosyltransferases for sLe^x-*O*-glycan precursor substrates, such as ST3Gal-I. In addition, B cells have low or no expression of the relevant E-selectin binding protein scaffolds, such as PSGL-1, CD43 and CD44. All together, these results may explain the absence of E-selectin binding activity of peripheral B cells.

Collectively, our findings offer novel perspectives on the functional and structural diversity of E-selectin ligands that are expressed by different mononuclear blood subsets and, moreover, may contribute to a greater understanding of the kinetics of leukocyte

recruitment into inflamed tissues during acute or chronic inflammatory conditions.

7.2 Strategies to improve the effectiveness of DC-based therapy

Dendritic cell (DC)-based vaccines represent a promising and a novel immunotherapy for treatment of cancer.[26] DCs are the most effective antigen presenting cells (APCs) and are crucial coordinators of both innate and adaptive immune systems. These cells are able to elicit both *naïve* and memory antigenic-specific immune responses, and therefore, they are extremely important in the induction of anti-tumor specific cytotoxic responses.[27,28] However, DC-based vaccination have not yet been shown to translate into clinical progress. Indeed, since the first DC-based vaccine clinical trial, in the mid-90s, multiple trials have been piloted within an extensive range of malignancies; though, only a small percentage of patients responded to the therapy with improved clinical outcome.[29] Thus, there is an urgent need to develop new technologies that are able to improve the efficacy of DC-based immunotherapy.

7.2.1 Elucidating the E-selectin ligands expressed by DCs: A contribution to the development of DC-based vaccines

An immediate hurdle in achieving the promise of DC-based therapeutics is the safe delivery of these cells from vasculature to target tissue, where they can exert their immunological functions.[26]

For clinical purpose, human DCs are most commonly obtained from differentiation of human blood monocyte precursors (mo-DCs),[30] following CD14 immuno-magnetic selection (CD14-S) or by plastic adherence of monocytes (PA-S).[31] Therefore, **in chapter 4**, we investigated the nature and the biological functions of the chief molecular effectors involved in transendothelial migration of human mo-DCs obtained from both PA-S and CD14-S methods. Surprisingly, our results show that CD14-S mo-DCs display a significant greater transendothelial migration than PA-S mo-DCs; moreover, this process is dependent on E-selectin binding and VLA-4 expression. Notably, this differential ability to transmigrate is due to a distinctive expression of E-selectin reactive glycoproteins. While PA-S mo-DCs only express the glycoform of PSGL-1 (i.e. CLA), CD14-S mo-DCs express the E-selectin binding glycoform of CD44 (i.e. HCELL), in addition to CLA. Moreover, the expression of HCELL

by CD14-S mo-DCs and its engagement by vascular E-selectin provide them a unique ability to transmigrate circumventing chemokine-mediated signaling, i.e. the binding of HCELL (and not CLA) to E-selectin directly activates VLA-4 adhesiveness to endothelium. Although, this mechanism has been described in human mesenchymal stem cells following enforced HCELL[25,32], our findings are the first to show that chemokines are not essential for human DC ability to transendothelial migrate. In addition, our results show that TNFα maturation of CD14-S mo-DCs induces maintenance of HCELL expression, which otherwise undergoes time-dependent decay. This is extremely pertinent because the cytokine TNFα is one of the cytokines mostly used to mature DCs for immunotherapeutics.[33] Lastly, we observed that exofucosylation of CD14-S mo-DC significantly increases expression of HCELL, and thereby augment their ability to undergo transendothelial migration.

Overall, we report that the expression of HCELL by mo-DCs licenses these cells to transmigrate without chemokine input; HCELL expression on mo-DCs can be achieve by generating mo-DCs via CD14-S method and maintained by TNF-α maturation, whereas exofucosylation increases its expression and therefore improves DCs transendothelial migration. Furthermore, the use of PA-S mo-DCs in adoptive DC-based immunotherapy may negatively affect DC tissue migration, reducing their efficacy.

In **chapter 5**, we performed a detailed analysis of the expression of E-selectin ligands on human monocyte-derived DCs and murine DCs obtained from different cellular sources.

Although human monocytes are typically obtained from peripheral blood, there has been an increasing clinical interest in monocytes obtained from umbilical cord blood source (UCB).[34] Therefore, we compared the E-selectin ligand expression on human mo-DCs obtained from these two monocytic cellular sources. Strikingly, we observed that human mo-DCs generated from differentiation of UCB monocytes completely lack E-selectin reactive glycoproteins. Yet, our data suggest that UCB mo-DCs display E-selectin ligand determinants in lipid scaffolds. Contrariwise, adult peripheral blood (APB) mo-DCs display a wide repertoire of E-selectin binding glycoproteins. As it was reported in **chapter 4**, these cells, which were generated by CD14-S method, express the E-selectin reactive glycovariants of PSGL-1 (CLA) and of CD44 (HCELL). Moreover, in **chapter 5** our data show that the glycoform of CD43 (i.e. CD43E) is also a functional E-selectin ligand expressed by APB CD14-S mo-DCs. Additionally, enforced exofucosylation of APB not only augment the expression of functional

CLA, HCELL and CD43E, but also induce the creation of the newly E-selectin reactive glycoform of CD18 and of high molecular weight glycoforms of HCELL. This finding is extremely pertinent, since CD18 is the β-chain of CD18/CD11 integrins.[35] CD18/CD11 integrins, along with β1 integrins, are the main mediators of firm adhesion of leukocyte to endothelium during extravasation process. Therefore, our findings may suggest a new role for CD18 in DC extravasation process.

Finally, our data also show a differential expression of E-selectin binding structures among murine DCs obtained from different cellular sources: bone marrow derived DCs (BM-DCs) and murine splenic DCs (sDCs). While, murine BM-DCs are devoid of E-selectin reactive glycoproteins, sDCs strongly express CLA.

Collectively, our findings disclose critical and undiscovered cellular sources-specific and species-specific diversity in the expression of E-selectin ligand glycoproteins among DCs. Importantly, our data suggest that monocytes isolated from APB may be a better source for *ex vivo* generated anti-cancer DC-based vaccines since APB mo-DCs natively display high contents of E-selectin ligands, which are required for DC migration to inflamed/tumoral tissues.

7.2.2 Immune-modulation of DC functionality via sialic acid removal

Another reason for the limited efficacy of DC-based vaccines in inducing an effective anti-tumor response in the treated patients is the insufficient maturation state of DCs used in the vaccination protocols.[26] Indeed, effective DC maturation implies three signals: capacity to present antigens via MHC (signal 1), co-stimulation (signal 2) and expression of pro-inflammatory cytokines (signal 3).[36] Nevertheless, there is no satisfying method to induce simultaneously all three aspects of maturation. Thus, in **chapter 6**, we described a new methodology that can be used to induce fully maturation of DCs.

DCs show a specific glycan profile at cell surface that can be modulated during development, maturation and immune regulation.[1] Specifically, they exhibit high levels of surface sialic acids, which are known to be associated with an immature and tolerogenic phenotype.[37-39] Therefore, we sought to evaluate the effect of sialic acid removal from DC surface on their maturation profile and consequent ability to induce specific anti-tumor immune responses.

Using both human and murine DCs, we observed that desialylation of DCs induced a higher DC maturation phenotype, via the upregulation of MHC and co-stimulatory molecules and through the secretion of Th1 cytokines, such as IL-12. The secretion of IL-12 by DCs is extremely critical for the initiation of anti-tumor immune responses, since this cytokine is the main inducer of Th1 immune responses, and consequently, of the generation of cytotoxic $CD8^+$ T cells.[40] Consistently, we observed a higher ability of desialylated DCs to induce proliferation, activation and differentiation of T cells into a Th1 profile; and, notably, to cross-present antigens, which resulted in increased priming and activation of antigen-specific $CD8^+$ T cells. This fully matured phenotype of desialyated human and mouse DCs translated into a higher ability of these cells to induce specific and potent cytotoxic responses against cancer cells. We consistently observed a higher ability of T cells that were primed with desialylated DCs to specifically kill tumor cells.

Collectively, our data demonstrate that desialylation of DCs is able to induce all the phenotypical and functional features of maturation, namely: (1) increased antigen presentation, (2) increased co-stimulatory ability and (3) secretion of Th1 cytokines. Moreover, desialylation of *in vitro* generated DCs elicits cross-presentation of tumor antigens, which is an essential mechanism for the induction anti-tumor cytotoxic T cell immune responses. Thus, sialidase treatment of DC surface is a useful tool to generate DCs with high capacity to induce activation of potent and highly effective anti-tumor-specific cytotoxic T cell-mediated immunity.

It is anticipated that the findings presented in the **chapters 4, 5 and 6** will provide fundamental insights that will guide the development of novel therapeutic strategies using DCs to treat cancer.

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Chapter 8

Other contributions

Other contributions:

During this PhD, I have also been involved in a project, which aim is to investigate the role of the sialylated-tumor antigens on the malignant phenotype of bladder cancer cells, on response to BCG therapy and immune mediated responses.

My participation on the mentioned study contributed to the following scientific papers:

As co-first author:

Severino P. F. and **Silva M.,** Carrascal M., Malagolini N., Chiricolo M., Venturi G., Astolfi A., Catera M., Videira P. A. and Dall'Olio F., 2014. Bacillus Calmette Guérin (BCG) therapy in bladder cancer: are the glycosidic antigens sialyl-T and sialyl-Tn main regulators? An in vitro approach. *Submitted to Molecular Cancer*

As second author:

Lima L., Severino P. F., **Silva M**., Miranda A., Tavares A., Pereira S., Fernandes E., Cruz R., Amaro T., Reis C. A., Dall'Olio F., Amado F., Videira P. A., Santos L. and Ferreira J. A., 2013. Response of high-risk of recurrence/progression bladder tumours expressing sialyl- Tn and sialyl-6-T to BCG immunotherapy. Br J Cancer 109, 2106-14.

Carrascal M. A., Severino P. F., Cabral M. G., **Silva M**., Ferreira J. A., Calais F., Quinto H., Pen C., Ligeiro D., Santos L. L, Dall'Olio F. and Videira P. A., 2014. Sialyl Tn-expressing bladder cancer cells induce a tolerogenic phenotype in innate and adaptive immune cells. Mol Oncol published online.

Ferreira J. A., Videira P. A., Lima L., Pereira S., **Silva M**., Carrascal M., Severino P. F., Fernandes E., Almeida A., Costa C., Vitorino R., Amaro T., Oliveira M. J., Reis C. A., Dall'Olio F., Amado F. and Santos L. L., 2013. Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours. Mol Oncol 7, 719-31.

Book chapter:

Severino P., **Silva M**., Carrascal M., Calais F., Dall'Olio F. and Videira P., 2012. Bladder cancer-glycosylation insights, Vol. 38, The Royal Society of Chemistry, pp. 156-175

TITLE

***Bacillus Calmette Guérin* (BCG) therapy in bladder cancer: are the glycosidic antigens sialyl-T and sialyl-Tn main regulators? An in vitro approach.**

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ABSTRACT

Background. Sialylated forms of truncated sugar chains *O*-linked to glycoproteins, known as Thomsen-Friedenreich-(TF) related antigens are aberrantly expressed in bladder cancer (BC). Treatment with *Bacillus Calmette-Guérin* (BCG) is an effective adjuvant immunotherapy of BC but still one third of the patients do not respond to treatment.

Methods. To investigate the role of sialylated TF antigens on BC biology and BCG response, the human BC cell lines HT1376 and MCR were retrovirally transduced with sialyltransferases *ST3GAL1* or *ST6GALNAC1* respectively, to obtain the cell lines $HT1376_{sT}$ and MCR_{sTn} or with empty retroviruses to obtain the negative controls $HT1376_{Nc}$ and MCR_{Nc}. Cytokines secreted by transduced BC cell lines challenged with BCG and by macrophages stimulated with the conditioned medium of BCG-challenged BC cell lines were measured by multiplex immune-beads assay. Global gene expression in sialyltransferase-transduced cell lines was measured after BCG challenge by expression microarray. Cell cycle was studied by FACS analysis of propidium iodide stained cells. Student's t test was used for statistical analysis. **Results.** After BCG-challenge, $HT1376_{sT}$ secreted more IL-8 while MCR_{sTn} secreted more IL-8 and IL-6 than their negative controls $HT1376_{Nc}$ and $MCR_{Nc.}$ Soluble factors secreted by BCG-challenged BC cells strongly increased the release of cytokines IL-6, TNF-α, IL-1β and IL-10 from macrophages. The stimulatory effect was higher with soluble factors from $HT1376_{sT}$ and MCR_{sTn} cells, showing that BCG response was dependent on the expression of sialylated TF antigens. Microarray analysis revealed that hundreds of genes were modulated by either sialyltransferase, mainly toward a putatively more malignant phenotype. The down-regulation of several genes involved in preserving genomic stability in both $HT1376_{sT}$ and MCR_{sTn}, resulted in signs of cell cycle alterations with accumulation of cells in the phases S and G2. BCG challenge resulted in a stronger modulation of the transcriptome in $HT1376_{sT}$ and MCR_{sTn} cells than in their negative controls with a tendency, in MCR_{sTn} cells, to increased expression of regulatory RNA genes.

Conclusions. Sialylated TF antigens may turn BC biology toward a more malignant phenotype but, because of their ability to enhance the response to BCG treatment, their expression might be a favourable predictor of successful adjuvant therapy.

Keywords

adjuvant therapy; Bacillus *Calmette-Guérin;* bladder cancer; gene expression analysis; glycosylation; Sialylated antigens; sialyltransferase; Thomsen-Friedenreich antigens;*;*

Introduction

Bladder cancer (BC), one of the most common cancers in Europe, is commonly treated with the intravesical inoculation with the *Bacillus Calmette-Guérin* (BCG), as an adjuvant therapy after surgery. In spite of its efficacy, a significant number of patients fail to respond to this therapy. Although the precise mechanisms involved in BCG anti-tumour activity remains uncertain, it is clear that BCG induces a local immune response activation [1-3]. Thus, a comprehensive understanding of how BCG can modulate the immune system and induce phenotypic changes of BC can help to further define therapeutic targets and improve patient's treatment.

Glycosylation is one of the most frequent post-translational modification of proteins, which undergoes profound changes in all types of cancer [4], including BC [5-8]. The biosynthesis of the sugar chains of glycoconjugates is mediated by glycosyltransferases, a class of enzymes which usually transfer a sugar from a sugar-nucleotide donor to a carbohydrate chain acceptor. Specifically, the sugar sialic acid is transferred by sialyltransferases, usually to the termini of the sugar chains. Thomsen-Friedenreich (TF)-related antigens are a group of small sugar chains *O*-glycosidically linked to serine or threonine, which includes Tn (GalNAc-*O*-Ser/Thr), T (Galβ1,3GalNAc-*O*-Ser/Thr) and the respective sialylated forms: sialyl-Tn (sTn, Siaα2,6GalNAc-*O*-Ser/Thr) and 3'sialyl-T (sT, Siaα2,3Galβ1,3GalNAc-*O*-Ser/Thr) antigens. These antigens are aberrantly expressed in a variety of cancers, including BC [7-13] and play a key role in cancer progression, affecting the invasive potential and the immune recognition [14-16].

The sT antigen is present in the healthy urothelium but is overexpressed in BC, due to an increased expression of sialyltransferase ST3GAL1 [11]. The sTn antigen is expressed in 75% of high grade bladder tumours but it is not observed in normal urothelium. The expression of sTn is correlated with an overexpression of the sialyltransferase ST6GALNAC1 [7, 17]. Recent data indicated that BCG immunotherapy was more efficient against tumours expressing sTn [8].

In this study, we investigated the consequences of the overexpression of sialyltransferases *ST3GAL1* and *ST6GALNAC1* and of their cognate sugar structures sT and sTn on the transcriptome modulation of BC cells and their ability to activate the innate immune system in response to BCG challenge.

Results

Characterization of sialyltransferase-modified cell lines.

To understand the role of sT and sTn in BC, we first developed cell line variants overexpressing these antigens. The BC cell lines HT1376 and MCR were chosen for transfection with sialyltransferases *ST3GAL1* or *ST6GALNAC1* respectively, because of their native low expression of the two sialyltransferases and of sT or sTn antigens [11]. To obtain cell populations with higher and more homogeneous expression of sT or sTn antigens, after transfection with *ST3GAL1* or *ST6GALNAC1*, two different strategies were used: clonal selection for $HT1376_{sT}$ cells and magnetic sorting for MCR_{sTn}. In the case of sT-expressing HT1376 clones, clonal selection was used due to the lack of a tool directly recognizing the sT antigen and the expression of sT antigen was indirectly deduced from the decreased reactivity with the T-specific lectin PNA. The resulting cell lines, comprised of more than 95% of cells either not showing PNA reactivity or expressing sTn, respectively are hereafter referred to as HT1376sT and MCRsTn, while their respective negative controls are $HT1376_{Nc}$ and MCR_{Nc}.

The activities of ST3GAL1 and ST6GALNAC1, in HT1376 and MCR cells were measured by transfer of sialic acid to exogenous acceptors: benzyl-T or asialo-BSM, respectively (Figure. 1A). The activity of either transduced sialyltransferase was highly expressed only in $HT1376_{sT}$ and MCR_{sTn} cells, while in $HT1376_{Nc}$ and MCR_{Nc} it was just above the background. As expected, the mRNA level of *ST3GAL1* and *ST6GALNAC1* was also highly expressed in $HT1376_{sT}$ and MCR_{sTn} cells (Figure 1B), while in the respective control cells the two transcripts were nearly undetectable. It is worth mentioning that the level of expression of transduced *ST6GALNAC1* was about 20 fold higher than that of transduced *ST3GAL1*.

The changes in the pattern of carbohydrate structures induced by the overexpression of the two sialyltransferases was investigated using PNA and anti-sTn antibody staining (Figure 1C), before and after sialidase treatment, which removes sialic acids. *ST3GAL1* overexpression in HT1376sT cells induced a dramatic decrease of PNA reactivity, compared to $HT1376_{Nc,}$ which was completely reversed by sialidase treatment, indicating that the masking of T antigens was mainly due to the ST3GAL1-mediated addition of α2,3-linked sialic acid. No sTn antigen was detectable in $HT1376_{Nc}$ or $HT1376_{sT}$ cells.

ST6GALNAC1 overexpression in MCR cells induced the expected increase of sTn antigen, which was almost completely reversed by sialidase treatment (Figure 1C). However,

ST6GALNAC1 overexpression in MCR cells also led to a substantial decrease of the T antigen, as demonstrated by the decreased reactivity to PNA. This effect could be explained considering that, according to its known substrate specificity [18], ST6GALNAC can mediate the α2,6-sialylation also of T antigen and of sT antigen, resulting in the biosynthesis of sialyl-6-T and diasialyl-T antigens (Fig. 1 D). These two antigens would not be recognized by PNA. Sialidase treatment induced a remarkable increase of PNA-reactivity in both MCR_{Nc} and MCR_{sTn} cells. This finding demonstrated that in the former the masking of the T antigen was due to α2,3-sialylation of galactose (sT antigen), while in the latter the masking of the T antigen was probably due to both α2,3- and α2,6-sialylation.

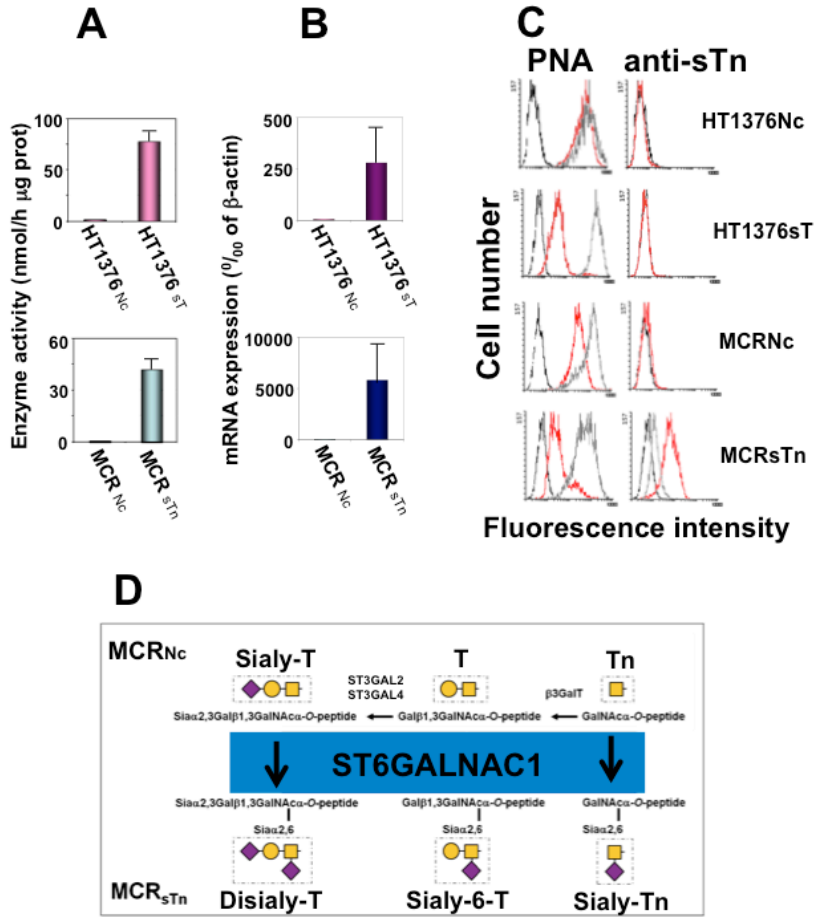

Fig. 1. Characterization of sialyltransferase-modified BC cell lines. $HT1376_{sT}$, MCR_{sTn} cells and their respective negative controls $HT1376_{Nc}$, MCR_{Nc} were characterized with respect to sialyltransferase activity (A), mRNA expression (B) and reactivity with PNA or anti sialyl-Tn (C). A: activity of ST3GAL1 and ST6GALNAC1 was measured by using benzyl-T and asialo-BSM as acceptors, respectively. Data are the mean ± SD of three experiments. B: Transcript abundance was measured by real time RT PCR and expressed as sialyltransferase mRNA molecules/1000 b-actin molecules. Data are the mean ± SD of three experiments. C: PNA and anti-sTn antibody reactivity was investigated by FACS. Black line: unlabelled cell lines when labelling with lectins or isotype control antibody, in case anti sTn antibody was used. Red line: cell lines without sialidase treatment. Grey line: cell lines with sialidase treatment. D: Diagram showing that ST6GALNAC1 acts

not only on Tn antigen but also on T and sT structures, potentially giving rise to their α2,6-sialylated counterparts and explaining PNA-reactivity data in MCR_{Nc} and MCR_{sTn}. GalNAc: yellow square; Gal: yellow circle; Sia: purple diamond.

Cytokine secretion by bladder cancer cell lines

We next sought to assess whether the expression of *ST3GAL1* or *ST6GALNAC1* would have an effect on cytokine production by BC cells following BCG stimulation. For that, we evaluated the levels of several cytokines in conditioned medium of BC cells, challenged or not with BCG. Of the following cytokines: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IFN-γ and TNF-α, only IL-6 and IL-8 were detectable. HT1376 cells produced little or no IL-6, which significantly increased after BCG stimulation, independently on the expression of ST3GAL1 (Figure 2). HT1376 natively produced high levels of IL-8, which increased further after BCG stimulation. Interestingly, the secretion of IL-8 was significantly higher in HT1376sT than HT1376Nc, after BCG challenge.

MCR cells natively produced high levels of IL-6, which significantly increased after BCG stimulation, only in MCRsTn cells (Fig. 2). In contrast, secretion of IL-8 by MCR was almost negligible but dramatically raised after BCG stimulation. Secretion of IL-8 after BCG stimulation, was also significantly higher in MCR_{sTn} cells than in negative control MCR_{Nc} (Fig. 2).

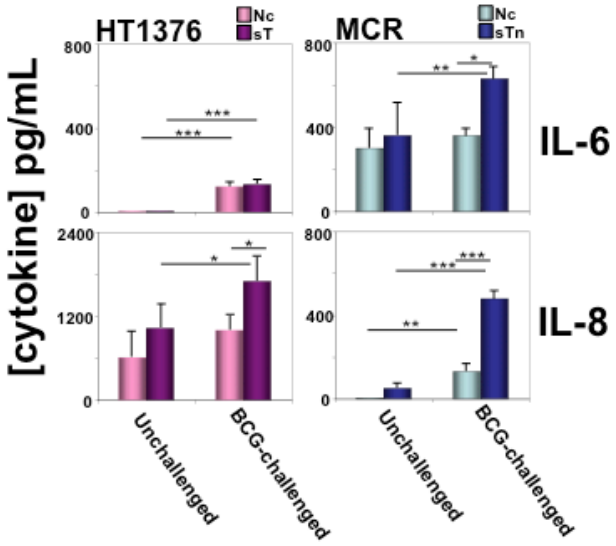

Fig. 2. Cytokine secretion by BCG-challenged BC cell lines. IL-6 and IL-8 were secreted by $HT1376_{Nc}$, (light violet) and $HT1376_{sT}$ (dark violet), and by MCR_{Nc} (light blue) and MCR_{sTn} (dark blue) cells. Cells were

challenged with BCG for 2 hours, then they were washed and cultured in medium for 16 hours. Cytokine concentration was determined as described in Materials and Methods. Data are the mean ± SD of three experiments. ***p<0.0001; **p<0.001; *p<0.05, according to Student's t test.

Cytokine secretion by human macrophages stimulated by conditioned medium from BC cells

To investigate the role played by BCG-stimulated BC cells on the first line innate immunity, the effect of the conditioned medium of BCG-challenged BC cells on human macrophage response was evaluated. The secretion of cytokines was measured in the culture medium of macrophages stimulated or unstimulated for 24 hours with the conditioned medium of BCG-challenged or unchallenged BC cell lines (Figure 3). IL-8 was the only cytokine secreted at high levels by unstimulated macrophages, while IL-1β and IL-10, although clearly detectable, were produced at a very low level. Macrophages stimulated with conditioned medium from unchallenged HT1376 or MCR cells did not present a robust cytokine secretion. However, the conditioned medium from BCG-challenged BC cells induced a significant secretion of IL-6, IL-1β, TNF-α and IL-10 (Fig. 3). Interestingly, the induction of cytokine secretion was markedly stronger when BCG-challenged cells expressed either sialyltransferase (Fig. 3). The cytokines IL-2, IL-4, IL-12, and IL-17 were not expressed in our experiments, in any condition (data not shown).

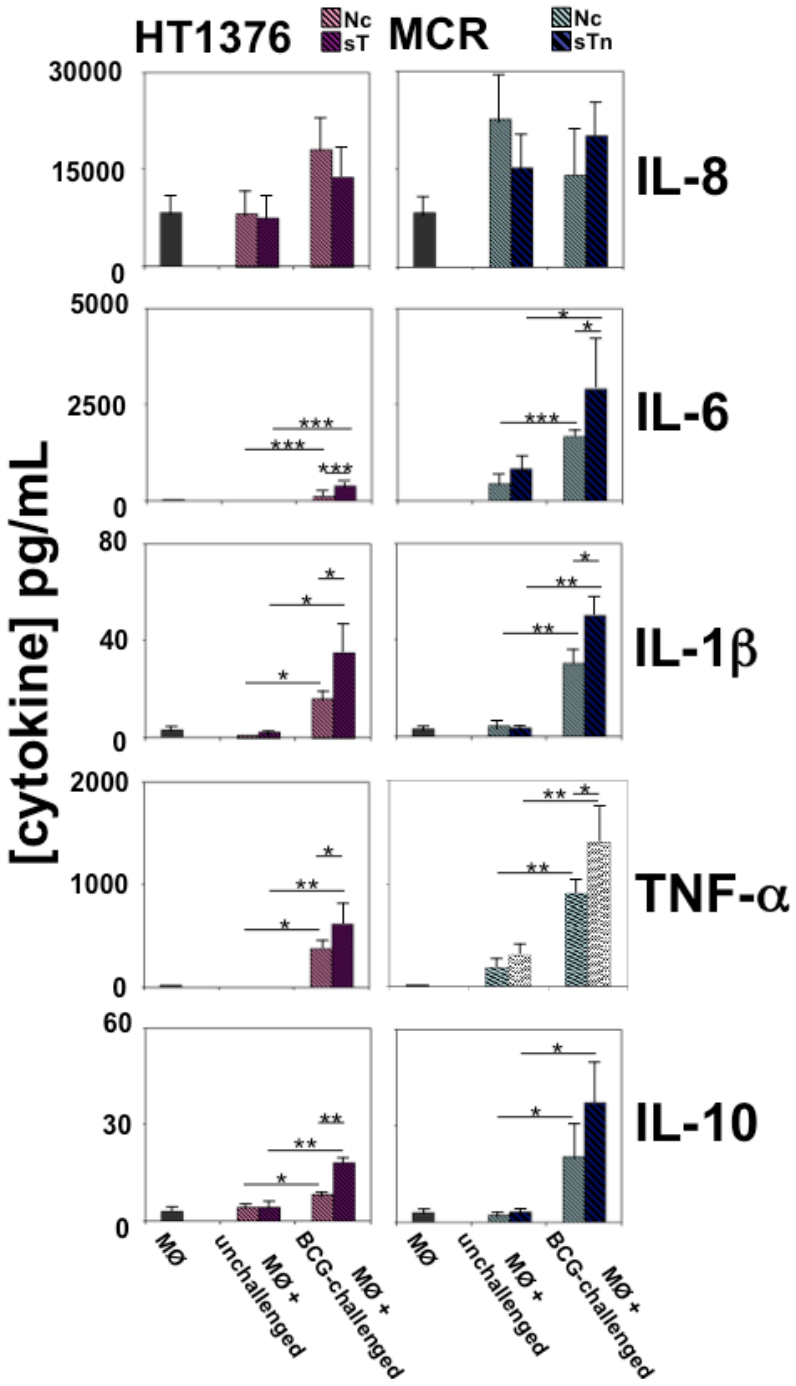

Fig. 3. Cytokine secretion by human macrophages treated with conditioned media of bladder cancer cell lines. Cytokine secretion was measured in culture media conditioned by unstimulated macrophages (gray bars) or stimulated with conditioned media from BCG-challenged or unchallenged BC cell lines: $HT1376_{Nc}$, (light violet); $HT1376_{sT}$ (dark violet); MCR_{Nc} (light blue); MCR_{sTn} (dark blue). BC cells were challenged with BCG for 2 hours, then they were washed and allowed to condition the culture medium for 16 hours. The conditioned culture media of BCG-challenged or unchallenged BC cell lines was used to stimulate human monocyte-derived macrophages for 24 hours. MØ: cytokines released by unstimulated macrophages; MØ + untreated: cytokines released by macrophages stimulated with the medium of unchallenged BC cell lines; MØ + BCG-treated: cytokines released from macrophages stimulated with the medium of BCG-challenged BC cell lines.

Macrophage stimulation by unchallenged HT1376 or MCR cells was negligible. With the exception of IL-8, the expression of either sialyltransferase in by BCG-challenged BC cells potentiated cytokine release by macrophages. Data are the mean ± SD of 3 experiments. ***p<0.0001; **p<0.001; *p<0.05. according to Student's t test.

Effect of sialyltransferase overexpression on the transcriptome of BC cell lines

The overall impact of the expression of *ST3GAL1* or *ST6GALNAC1* sialyltransferases on the two BC cell lines was evaluated by analysing the transcriptome changes by expression microarray technology. Data summarized in Figure 4A refer to genes modulated at least by a $\log_2$ expression ratio ≥ 1.0 (meaning a fold change of at least 2) and show the up- or down-regulation of genes induced by the expression of either sialyltransferase in the two cell lines. Both ST3GAL1 and STGALNAC1 expression induced in HT1376 or MCR significantly changed the expression of hundreds of genes. In particular, 448 genes (excluding the transduced gene) were modulated in HT1376 (187 up-regulated and 261 down-regulated) while 488 genes (excluding the transduced gene) were modulated in MCR cells (185 up-regulated and 303 down-regulated) (Fig. 4A). The expression level of the transduced genes determined by microarray technology confirmed that expression of ST3GAL1 and of ST6GALNAC1 was much higher in $HT1376_{sT}$, and MCR_{sT} cells than in respective control (data not shown). In addition, the increase of ST6GALNAC1 expression was higher than that of ST3GAL1, as previously determined with real time PCR (Figure 1B). A comparison of the level of expression of the two transduced genes by microarray and by real time PCR indicated that microarray data lead to underestimate the differences in comparison with real time PCR.

The genes modulated by the two sialyltransferases are involved in several specific cell functions. Owing to the well-known role of sT and sTn in cancer growth, we have focused the analysis on genes with a recognized role in tumour growth and progression. We have identified 33 modulated genes in HT1376 cells and 21 in MCR whose up- or down-regulation could, according to literature data, play a putative role in promoting or inhibiting cancer growth. Expression of ST3GAL1 in HT1376 cells induced the change towards increased malignancy of 19 cancer genes and toward decreased malignancy of 14 cancer genes (Fig. 4B). The cellular functions of the cancer genes modulated toward increased malignancy by *ST3GAL1* in HT1376 cells include the regulation of apoptosis (*FRMD3*), of cell growth (*TGFB2, LOXL4*), of angiogenesis (*THBS1*) or proteolysis (*ALCAM, SERPINE2*). A group of twelve genes involved in preserving mitotic fidelity and chromosomal stability or involved in

different DNA repair mechanisms, such as homologous recombinational repair (HR) and nucleotide excision repair (NER), which can be collectively referred to as "caretaker genes", displayed down-regulation in $HT1376_{sT}$ cells (Table 1). However, if also genes with a modulation log_2 expression ratio ≥ 0.5 were considered, the number of down-regulated caretaker genes in $HT1376_{sT}$ cells reached 28. The most remarkable changes within the group of 14 genes modulated toward putatively decreased malignancy in $HT1376_{sT}$ cells, were found in genes directly involved in the control of cell growth (*SH3BGRL, CCND1, TPTE),* inflammation *(AIM2* and *SLC39A2*), proteolysis (*SERPINE1, ADAM12*) and angiogenesis (*OLFML3*).

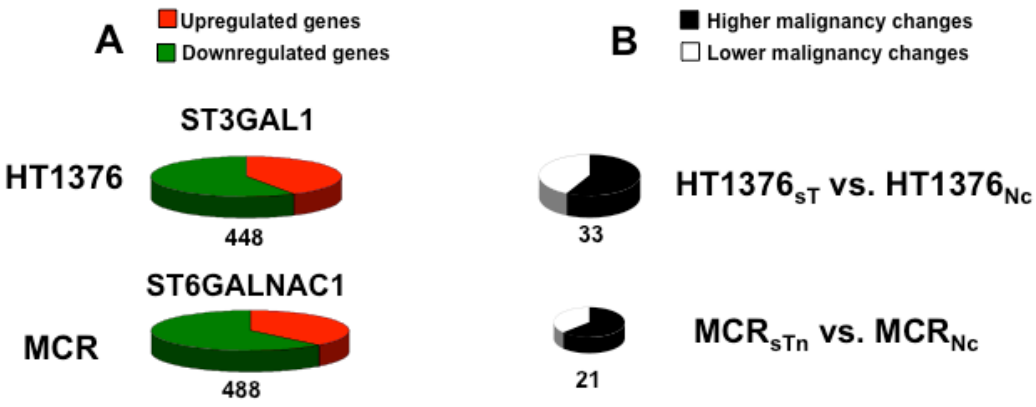

Fig. 4. Impact of sialyltransferase expression on gene expression in BC cell lines. The gene expression was evaluated in HT1376 and MCR cell lines by expression microarray technology. A: Number of genes which underwent up- (red) or down-regulation (green) after *ST3GAL1* expression in $HT1376_{sT}$ and after *ST6GALNAC1* expression in MCR_{sTn} cells, compared to their respective negative control $HT1376_{Nc}$ and MCR_{Nc}. The area of the two graphs is proportional to the number of modulated genes, which is reported below each graph. B: Number of genes modulated by either sialyltransferase whose changes putatively lead to increased (black) or decreased (white) malignancy, according to literature. The area of the two graphs is proportional to the number of modulated genes, which is reported below each graph.

In MCR_{sTn} cells, compared to MCR_{Nc}, the proportion between cancer genes modulated toward increased or decreased malignancy was 14 to 7, respectively. The cellular functions of the former include apoptosis (*GLIPR1*), cell growth (*GPR87, LOX, ERBB4, LPAR6*), angiogenesis (*JAM3*). The most remarkable changes of the cancer-associated genes modulated toward decreased malignancy in $MCRs_{Tn}$ include *PLCB1* and *STC1* whose products stimulate cell growth, *PLEK2* involved in cytoskeletal rearrangements and genes *MME, SERPINE1* and *SERPINE2*, involved in proteolytic activity. Like in $HT1376_{sT}$, in

$MCRs_{Tn}$ a group of 5 caretaker genes, (which became 13 when a $\log_2$ expression ratio ≥ 0.5 was used) was down-regulated compared to MCR_{Nc} (Table 1).

It is expected that the co-ordinate down-regulation of genes involved in DNA repair and mitotic fidelity leads to alterations of the cell cycle, in particular to increased percentage of cells in S and G2/M phases and irregular shape of the G0 + G1 peak, as observed in case of chromosomal instability [19]. In a typical cell cycle analysis experiment (Fig. 5) the percentage of cells in phases S + G2/M of the cell cycle raised from 17% in $HT1376_{Nc}$ to 28% in $HT1376_{sT}$ and from 28% in MCR_{Nc} to 33% in MCR_{sTn}. Moreover, the G0 + G1 cell population of $HT1376_{sT}$ was contained in a broader and less regular shaped peak than the corresponding peak of $HT1376_{Nc}$. These alterations are consistent with the predicted effects of the down-regulation of genes involved in DNA repair and mitotic fidelity.

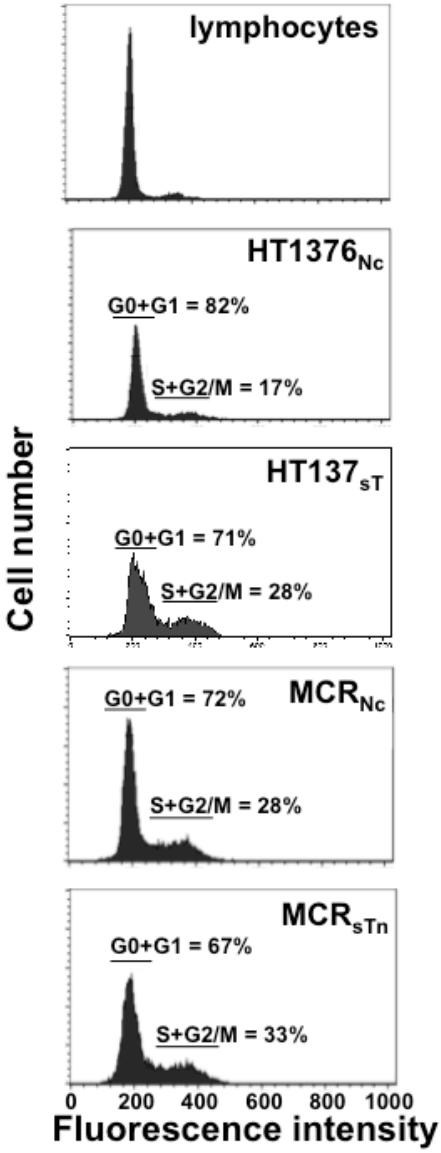

Fig. 5. FACS analysis of the cell cycle of BC cell lines. Cells were stained with propidium iodide and FACS analyzed. Data were acquired in linear scale. Normal human lymphocytes were used as reference of a 2N resting cell population in G0/G1 phases of the cell cycle (region under the left bar). The region under the right bar contains cells with a >2N DNA content corresponding to S + G2/M phases of the cell cycle. All four BC cell lines gave a major peak of 2N cells and lower percentages of cells in S and G2/M phases. In both, $HT1376_{sT}$ and MCR_{sTn}. the percentage of cells in >2N phases was remarkably increased. Representative experiment of four.

Effect of BCG challenging on whole gene expression in BC cell lines

The effect of BCG challenging on global gene expression was evaluated by classifying genes modulated at least by a $\log_2$ expression ratio ≥ 1.0 (fold change of at least 2) in several broad functional categories (Table 2). BCG challenge on $HT1376_{Nc}$ resulted in up-regulation of 20 genes and down-regulation of 15 genes, while in $HT1376_{sT}$ the number of up-regulated and

down-regulated genes was 20 and 79, respectively. Surprisingly, there were no genes showing parallel and consistent up- or down-regulation in the two HT1376 cell variants using a threshold fold change ≥ 2.0, while many genes showed parallel regulation using a threshold fold change ≥ 1.4 (data not shown). ST3GAL1 expression made the HT1376 cell genome more susceptible to BCG modulation, with a higher number of down-regulated genes. In particular, we observed down-regulation of a set of genes related to "aminoacid and protein biosynthesis" only in $HT1376_{sT}$ (Table 2). This functional category includes genes involved in the biosynthesis and transport of aminoacids, attachment of aminoacids to tRNAs and regulation of translation. Genes involved in cell growth and survival, genome stability, intracellular transport, ionic homeostasis, lipid metabolism and transport, proteolysis and signal transduction were also more frequently modulated in $HT1376_{sT}$ than in $HT1376_{Nc}$. The majority of the genes involved in post-transcriptional regulation were RNA genes, such as small nuclear RNAs (snRNAs), *RNU2-1, RNU4-2, SCARNA9, RNU5B-1* involved in the regulation of splicing, and small nucleolar RNAs (snoRNA) *RNU5B-1, SNORD116-2, SNORA70B*, which are involved in chemical modification of other RNAs. Genes falling in the category of inflammatory and immune response were prevalently up-regulated in $HT1376_{Nc}$ and prevalently down-regulated in $HT1376_{sT}$. This category includes genes involved in antigen presentation (*HLA-DPB1)*, cytokine signalling (*IFNGR1, IL20RB)*, phagocytosis (*CD177*, *NCF1*), NK activity (*TXNIP, ULBP1).*

In the two MCR cell variants, BCG challenging resulted in modulation of a surprisingly low number of genes. The only gene expression change showing parallel modulation in MCR_{Nc} and MCR_{sTn} was the up-regulation of *HLA-DQA2*, encoding the α2 chain of major histocompatibility complex class II DQ, although in MCR_{sTn} the fold change was lower than 2. The functional category more affected by BCG challenging, but only in MCR_{sTn} cells, was “post-transcriptional regulation”. Eight of the nine genes of this category were snoRNA and one (*RNU5B-1*) was a snRNA. The vast majority of BCG-modulated genes displayed a fold-change between 2 and 3. Rarely genes were modulated at higher levels.

Discussion

Despite its effectiveness in reducing BC recurrence, treatment with BCG still comprises more than one third of non-responder patients. Recently, it was reported an association between

aberrant glycan (sTn antigen) expression by cancer cells and response to BCG [8]. This observation opened the possibility that glycosylation could be a predictive biomarker for response to BCG.

The overexpression of both ST3GAL1 [20, 21] and ST6GALNAC1 [22-25] has been widely studied in cellular model of breast cancer and found responsible for increased malignancy. However, very little is known on the role of these two enzymes in bladder cancer biology [7]. In this work we performed an exhaustive analysis of BC overexpressing ST3GAL1 or ST6GALNAC1 sialyltransferases resulting in the expression of the sT or the sTn antigens, respectively. We observed that in MCR cells the expression of ST6GALNAC1 led to both sTn expression and T-antigen inhibition, confirming that the expression of a glycosyltransferase induces the concomitant up-regulation of the cognate carbohydrate structure and the inhibition of alternative carbohydrate structures [26-29].

The secretion of cytokines [1, 30, 31] and the modulation of the expression of MHC molecules [32] are well known functional consequences of BCG interaction with BC cells. Our study confirms that BCG induces IL-6 and IL-8 secretion by BC cells and shows for the first time that the BCG response is strongly stimulated by the expression of sialylated TF antigens.

However, the role of inflammatory cytokines in cancer is often a double edge sword. In fact, both IL-6 [33-36] and IL-8 [37-40] can either promote or inhibit tumour progression supporting the notion that inflammation is useful to eradicate the tumour but also provides the necessary soil for cancer growth and progression. A key role in determining the outcome of the inflammatory response triggered by BCG-stimulated BC cells can be played by macrophages, which can be polarized to functionally different phenotypes, known as M1 and M2. The first, through IL-12 secretion, drives the polarization of T helper cells toward the inflammatory Th1 phenotype, while the second polarizes T helper lymphocytes towards the anti-inflammatory Th2 phenotype, which is thought to favour tumour growth [41]. However, M1 and M2 phenotypes are extremes in a continuum spectrum of functional conditions [41]. Our macrophages, which mainly secrete IL-6, IL-8 and TNF-α but little or no IL-12/IL-10 are probably representative of an intermediate condition. The phenotype of macrophages is of particular relevance in the light of the recent finding that correlated a worse response to BCG with the presence of M2 type macrophages [42]. Our data show that the release of inflammatory cytokines by macrophages was crucially dependent on their stimulation by the

conditioned medium of BCG-challenged BC cells expressing sialylated TF-related antigens. This may explain why the expression of sTn and of sialyl-6-T antigens (the biosynthesis of both is dependent on *ST6GALNAC1*) is predictive of BCG response and is associated with a better recurrence-free survival [8]. Thus, BCG treatment turns the expression of sTn/sialyl-6-T from an unfavourable condition associated with increased progression to a favourable condition associated with a better prognosis. This conclusion is of particular interest in the light of our recent observation that co-culture of MCR_{sTn} (not challenged with BCG) with dendritic cells has the effect of inducing a tolerogenic phenotype in dendritic cells and in T-lymphocytes [14].

Previous studies have shown that the gene expression profile undergoes profound changes in human BC, compared with urothelium [43] and in mouse urothelium after BCG treatment [44]. In this study we have assessed in the two BC cell lines the effect on whole gene expression of the forced sialyltransferase expression and the effect of BCG. The most prominent feature we observed as a consequence of the constitutive expression of *ST3GAL1* in HT1376 and of *ST6GALNAC1* in MCR cells was the decreased expression of several genes whose common feature is the preservation of genomic stability. In particular, we found down-regulated genes involved in different mechanisms of DNA repair and in the accuracy of chromosomal segregation during mitosis, suggesting that the expression of either sialyltransferase in the two cell lines can lead to forms of genetic instability. The alterations of the cell cycle we observed in the two sialyltransferase-transduced cell populations included the accumulation of cells in the S and G2/M phases of the cell cycle and, in $HT1376_{sT}$ cells, the irregular shape of the peak containing G0 + G1 cells. These alterations are consistent with a slow-down of the processes of DNA synthesis and mitosis, probably because of a more frequent activation of checkpoint mechanisms. Consistent with the reduced number of down-regulated "caretaker" genes, these alterations were less dramatic in MCR_{sTn} cells than in $HT1376_{sT}$ cells. However, none of the down-regulated "caretaker" genes was switched-off in either cell line, suggesting that the expected alterations would be milder that those described in frank chromosomal instability caused by the homozygous inactivation of APC gene in colon adenoma [19]. In both $HT1376_{sT}$ and MCR_{sTn}, several genes controlling cell growth and/or apoptosis displayed modulation. In MCR_{sTn} the changes toward increased malignancy were prevalent, in agreement with their reported slightly increased proliferation rate [7].

BCG challenging resulted in the modulation of a larger number of genes in HT1376 cells than in MCR cells. In both cell types, sialyltransferase-transduced cells were more prone to

BCG modulation, confirming that the presence/absence of sialylated TF-related glycans on the cell surface affects the development of the genetic program triggered by BCG contact. Even though genes involved in inflammatory and immune response were found modulated by BCG challenging in both HT1376 cell variants, little or no changes were observed in genes encoding MHC molecules and cytokines. This could depend on the sensitivity of the microarray technology, which is lower that other techniques, and/or on the role played by post-transcriptional mechanisms of control. This possibility is supported by the strong and consistent modulation of genes involved in post-transcriptional regulation. In particular, in both MCR cell variants the surprisingly low number of protein coding genes which were modulated was in sharp contrast with the high number of non-coding RNA genes which were up-regulated only in the MCR_{sTn} variant. The majority of these RNA genes belonged to the group of small nucleolar RNAs, which guide the methylation or pseudouridylation of other RNAs [45, 46], regulating protein expression. Through this mechanism, BCG could profoundly alter the pattern of protein expression of bladder cancer cells through a gene-expression independent mechanism.

Conclusions

In this study we have shown that the expression of sT and/or sTn antigens in BC cells induces changes of gene expression putatively affecting multiple cellular functions associated with increased malignancy. In particular, the down-regulation of genes responsible for genomic stability results in alterations of the cell cycle. However, the expression of sT and/or sTn antigens by BCG-challenged BC cells strengthen the inflammatory response of macrophages, indicating that the presence of these antigens can be a key factor for a successive BCG therapy and can be used as a predictive marker for therapy.

Materials and Methods

Cell lines

Both cell lines were from invasive transitional cell cancer of the bladder: HT1376 was from a primary tumour [47], while MCR was from a subcutaneous metastatic lesion [48]. Cells were grown in DMEM (4.5 g/L glucose, Sigma), containing 10% Foetal Calf Serum (FCS, Sigma), 2 mM *L*-glutamine (Sigma) and 100 μg/mL penicillin/streptomycin (Sigma).

Sialyltransferase-expressing cell lines

HT1376 cells expressing *ST3GAL1* and MCR cells expressing *ST6GALNAC1* were generated through lentiviral transduction, as described [7]. The transduced MCR cells were enriched in their sTn positive population (MCR_{sTn}), by magnetic-activated cell sorting (Miltenyi Biotec), using 1:50 diluted anti-sTn antibody (HB-STn1 clone, Dako) and magnetic sorting as recommended by the manufacturer.

HT1376 cells homogeneously negative for T antigen, according to negative reactivity to *Arachis hypogea* (PNA) lectin, ($HT1376_{sT}$) were obtained by selection of individual clones. About 100 transduced HT1376 cells were seeded in a 10 cm Petri dish and after one month nine colonies were randomly selected and the loss of the T antigen was detected by PNA-FITC staining by flow cytometry (FACS) analysis.

Flow cytometry

Cells were stained with the following lectins or antibodies. PNA was conjugated with fluorescein isothiocyanate (FITC) as previously described [49]. The anti-sTn antibody (TKH2 hybridoma clone) was kindly provided by Professor Celso Reis, University of Porto, Portugal. For anti-sTn staining, cells were incubated with 1:50 diluted TKH2 supernatant. , for 45 min at 4 ºC, and then with 1:100 diluted polyclonal anti-Ig-FITC (Dako). For PNA staining, cells were incubated with 1:20 diluted PNA-FITC. Cells were then washed and FACS analysed.

For the analysis of the cell cycle, cells were washed twice with PBS, gently suspended in ice-cold 70% ethanol and kept at 4 °C 16 h. Then cells were washed once by centrifugation, suspended with PBS containing 0.1% Triton X-100, 0.2 mg/mL DNAase-free RNAase and

0.08 mg/mL propidium iodide and incubated at 30 min at room temperature. Samples were FACS analyzed in linear scale.

Real time RT-PCR

Total RNA was isolated using the GenElute Mammalian Total RNA Purification kit and DNase treatment (Sigma), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed, using the random-primers based High Capacity cDNA Archive Kit (Applied Biosystems). The expression level of sialyltransferases *ST3GAL1* and *ST6GALNAC1* was evaluated with the TaqMan assay system (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems) using the TaqMan Universal PCR Master Mix Fast as described previously [11, 14, 50]. The assay identification and the detected reference sequences were the following*:* *ST3GAL1* (Hs00161688_m1; NM_173344.2; NM_003033.3); *ST6GALNAC1* (Hs00300842_m1; NM018414.2). The efficiency of the amplification reaction for each primer-probe was above 95% (determined by the manufacturer). The normalized mRNA expression was computed as ‰ of the endogenous control β-actin gene expression, calculated by using the $2^{-\Delta CT}\times 1000$ formula [51].

Sialyltransferase activity assays

Cell pellets were homogenized in water and the protein concentration of the homogenates was determined by the Lowry method. The activity of the two sialyltransferases was measured in the homogenates in the range of linearity with regard to time and substrate concentration as follows. ST3GAL1 activity was assayed as previously described [52] with some modifications. The reaction mixture contained in a 25 µL volume: 50 mM of 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer pH 6.5, 0.5% Triton X-100, 23.5 µg of Galβ1,3GalNAcα1-*O*-benzyl (benzyl-T; Sigma) as acceptor substrate, 15 µM (640 Bq) of CMP-[^{14}C]Sia (Amersham) and 50 µg of homogenate proteins. The enzyme reactions were incubated at 37 ºC, for 2 hours and the products were then isolated by hydrophobic chromatography in SepPak C18 Classic Cartridge (Waters). The columns were washed with water and eluted with 1 mL acetonitrile, which was counted in a liquid scintillation counter. The incorporation on endogenous substrates, prepared in the absence of the acceptor substrate, was subtracted.

ST6GALNAC1 activity was assayed as previously described [53] with some modifications. The reaction mixture contained in a 50 µL volume: 80 mM sodium cacodylate buffer pH 6.5, 0.5% Triton X-100 (Sigma), 0.3 mg of asialo bovine submaxillary mucin (asialo-BSM, prepared by acid desialylation of BSM as acceptor substrate, 30 µM (1280 Bq) of CMP-[^{14}C] Sia and 0.1 mg of homogenate proteins. The enzyme reactions were incubated at 37 ºC, for 2 hours, then the acid-insoluble radioactivity was measured in a liquid scintillation counter. The incorporation on endogenous substrates, prepared in the absence of the acceptor substrate was subtracted.

Sialidase treatment

Cells were treated with 20 mU of *Clostridium perfringens* sialidase (Roche Diagnostics), for 90 min at 37 °C and after washing with PBS by centrifugation, they were stained and FACS-analyzed as above.

BCG internalization by cell lines

Commercial Connaught strain BCG (ImmuCyst, Sanofi Pasteur SA, France) was suspended in PBS containing 0.05% Tween 80 and stored at -80 ºC. Before each assay, BCG aggregates were discarded by centrifugation (300 × g for 5 min). When required, about 1 O.D.$_{600nm}$ unit of single cell BCG was labelled with 2 µg/mL of orange cell tracker dye 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR, Invitrogen) for 2 hours in growth conditions, and incubated for other 2 hours in one volume of growing medium. BCG internalization by BC cell lines was accomplished by co-incubating cell lines with stained BCG in a BC cell:bacteria proportion of approximately 1:10. Cell lines, seeded in 24 wells plates, were incubated with 0.25 O.D.$_{600nm}$ units of BCG, at 37 °C for 2 hours. Internalization of stained BCG by cell lines was confirmed by FACS.

Preparation of monocyte-derived macrophages

Macrophages were obtained by differentiation of human monocytes from peripheral blood as follows. Blood from the Blood Collection Service of the Pizzardi Hospital of Bologna was diluted one fold with PBS and centrifuged at 200 × g, for 30 min at 20 ºC. The interface, containing the majority of the white blood cells, was diluted two folds with PBS and gently stratified in Ficoll-Hypaque (GE Healthcare) in a proportion of 5:3.7. Stratified preparations were centrifuged at 1200 × g for 30 min at 20 ºC and the white interface was carefully collected. This phase, containing the mononuclear cells, was washed abundantly with PBS, and cultured in RPMI 1640 (Sigma) medium supplemented with 20% FCS, 2 mM L-glutamine (Sigma) and 100 $\mu g.mL^{-1}$ penicillin/streptomycin (Sigma). Medium was daily renewed. After 7 days, monocyte-derived macrophages were detached with a cell scraper and dispensed in 24 well plates at approximately 50% of confluence for further assays.

Macrophage stimulation

BC cell lines were incubated with BCG as described above. Then the medium was removed, cells were washed twice with PBS and incubated with fresh medium without BCG. After 16 h, the conditioned media were collected and stored at -80 ºC, for further analysis. BC cells were washed, harvested and used for gene expression analysis. The media and cells mock treated in parallel without BCG were used as controls. The response of macrophages to components secreted by BCG-treated cell lines was assessed by incubating macrophages with conditioned media from BCG-challenged or unchallenged cell lines obtained as described above. After 2 hours, the media were changed and 24 hours later collected and stored at -80 °C for further analysis.

Detection of secreted cytokines

The content of cytokines was measured by enzyme-linked immunosorbent assay (ELISA) and multiplex immune-beads assay (MIBA). IL-6 was detected with a commercial 96 wells ELISA kit Raybiotech) as recommended by the manufacturer. After evaluation of IL-6, ten cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IFNγ and TNF-α) were evaluated in a 96-well strip plate from a commercial MIBA kit (Bio-Rad) as recommended by

manufacturer's instructions. In short, a mix of colour-coded micro beads coated with each specific antibody recognizing each cytokine were incubated with supernatant sample in a filtered bottom 96 wells MIBA plate, for 30 min at room temperature. Further steps of incubation with a mix of antibodies for 30 min and with Phycoerythrin-streptavidin (PE-streptavidin) for 10 min at room temperature were performed. Fluorescence was read in a Luminex 100 Bio-Plex Liquid Array Multiplexing System reader (Bio-Rad) and the data were analysed with the Bio-Plex Manager v5 software (Bio-Rad).

Whole transcriptome analysis by expression microarray

Total RNA was isolated by the guanidinium thiocyanate-method [54]. RNA was then converted to labelled single strand cDNA (ssDNA) by the commercial Whole Transcript Expression kit (Ambion), according to the manufacturer's instructions. Labelled ssDNA fragments virtually covering the whole transcriptome of transduced BC cell lines were then hybridized in a Human Transcriptome Array 2.0 overnight. Arrays were then washed and stained with phycoerythrin-streptavidin and fluorescence was read in a GeneChip Scanner 3000 7G (Affymetrix). Raw data were background-subtracted, normalised and summarised with the robust multi-array average (RMA) algorithm implemented in the Affy package of Bioconductor (http://www.bioconductor.org). Differential genes between query and control assay were selected by application of the *t*-test with a $p \leq 0.05$ cut-off and by the $\log_2$ expression ratio, considering only variations ≥0.5. Array data were analysed by the ArrayStar v2.0 software (DNASTAR) and through a literature search of the biological roles of modulated genes. Genes herein approached follow *HUGO Gene Nomenclature Committee* roules (http://www.genenames.org/), being represented in italic uppercase letters. With exception for cytokines, proteins herein discussed have the same name as the gene and are represented in regular uppercase.

Abbreviations: BC: bladder cancer; BCG: *Bacillus Calmette-Guérin;* BSM: bovine submaxillary mucin; CMTMR: 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; DMEM: Dulbecco's modified Eagle medium; FCS: foetal calf serum; FITC: fluorescein isothiocyanate; Gal: Galactose; GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglucosamine; MES: (*N*-

morpholino)ethanesulphonic acid; MIBA: multiplex immune-beads assay; PE: phycoerythrin; PNA: peanut agglutinin; RPMI: Roswell Park Memorial Institute; Sia: sialic acid; TF: Thomsen-Friedenreich

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FDO, PFS and PAV conceived the study, designed the experiments and wrote the manuscript. PFS and MS carried out the main experiments and analyzed data. M Carrascal, carried out experiments with cell lines and FACS analysis. NM contributed to enzyme assays and FACS analysis. M Catera and GV performed and analyzed data of FACS experiments. M Chiricolo contributed to cell culture experiments. AA performed microarray analysis and analyzed data. All authors read and approved the final manuscript.

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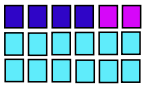
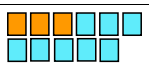
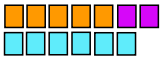
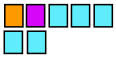
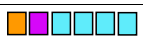
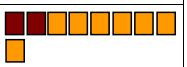
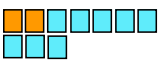
Tables

Table 1. Genes involved in maintaining chromosomal stability and/or DNA repair showing down-regulation in $HT1376_{sT}$ and/or MCR_{sTn} cells.

Down-regulated in $HT1376_{sT}$	Down-regulated in MCR_{sTn}	Function
CDCA5 ***[SGOL11]*** ***[HAUS6]*** ***HAUS7*** ***[HAUS8]*** *FRY* *KIF4A* *KIFC1* *BUB1B* *CCNF* *ORC1L* *SPC24* *CENP1* *[CENPF]* *[CENPM]* *[CDC20]* *[INCENP]*	***[CDCA5]*** ***SGOL1*** ***HAUS6*** ***[HAUS3]*** *[KNTC1]* *[CSPP1]*	Fidelity of chromosomal segregation during mitosis
[XRCC3] ***[XRCC6]*** ***[BRCA2]*** *RAD54* *[BRIP1]* *[EME1]* *[FANCA]* *[FANCC]* *[FANCD2]* *FANCG*	***XRCC4*** ***[XRCC2]*** ***BRCA1*** *[NBS]*	Members of homologous recombinational repair (HR)
[ERCC2]	***ERCC6*** ***[ERCC8]*** ***[ERCC6L]***	Members of nucleotide excission repair (NER)

"Caretaker" genes showing downregulation in either cell line or in both are grouped in 3 functional categories. In bold are indicated genes identical or closely homologues showing parallel down-regulation in both $HT1376_{sT}$ and $MCRs_{Tn}$. Genes in parenthesis displayed a fold change down-regulation between 1.4 and 1.9. The other genes displayed a fold change down-regulation ≥ 2.

Table 2. Modulation of gene expression by BCG challenging.

Functional Class	HT1376$_{Nc}$	HT1376$_{sT}$	MCR$_{Nc}$	MCR$_{sTn}$
Aminoacid and protein biosynthesis				
Cell adhesion				
Cell Growth and Survival				
Cytoskeleton Structure				
Drug Metabolism				
Energy Metabolism				
Genome Stability				
Inflammatory and Immune Response				
Intracellular Transport				
Ionic Homeostasis				
Iron Storage				
Lipid Metabolism and Transport				
Postranscriptional Regulation				
Proteolysis				
Signal Transduction				
Stress response				
Transcription regulation				
Ubiquitination				
Unclear function				

Genes showing a fold change ≥ 2 upon BCG challenging were classified in broad functional categories. Each square represents a modulated gene according to this code: up-regulation by BCG ≥ 4 (fold changes); up-regulation by BCG ≥ 3, <4; up-regulation by BCG ≥ 2, <3; down-regulation by BCG ≥ 4; down-regulation by BCG ≥ 3, <4; down-regulation by BCG ≥ 2, <3