Mechanisms by which Cell Surface Engineering of the Endothelial Glycocalyx with a Sialic Acid-Containing Polymer Prevents Organ Transplant Rejection

by

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Declaration of Committee

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Abstract

Organ transplant rejection is prevented by immunosuppressants, but these drugs do not always work and are associated with many side effects. To circumvent the use of these immunosuppressants, a cell surface engineering (CSE) approach that enzymatically adheres a sialic acid-containing polymer, LPG-Sia, to endothelial cells in blood vessels prior to transplantation was developed. I investigated the mechanisms by which this protocol inhibits immune cell activation. Co-culture assays showed that LPG-Sia decreased immune-mediated endothelial cell death. Blocking sialic acid receptors, siglec-7 and -9, prevented the protective effect of LPG-Sia indicating that they were required for immune inhibition. Transplantation of CSE modified allogeneic arteries reduced co-stimulatory molecule CD86 expression on recipient conventional dendritic cells. Also, accumulation of macrophages, CD4 and CD8 T cells were reduced in CSE modified allograft arteries. Together, these findings indicate that LPG-Sia deactivates immune cells by acting on siglec-7 and -9, and that it reduces early activation of antigen presenting cells that may prevent T cell activation and resultant rejection.

Keywords: Glycocalyx; sialic acid; siglecs; endothelial cell; transplant rejection

To my younger self

مل ہی جائے گی منزل بھٹکتے بھٹکتے

گمراہ تو وہ ہے جو گھر سے کبھی نکلی ہی نہیں

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List of Acronyms

APC	Antigen presenting cell
ApoB	Apolipoprotein B
BCR	B cell receptor
$CaCl_2$	Calcium chloride
CD#	Cluster of differentiation-#
CD22	Siglec-2
CD33	Siglec-3
CMP	Cytidine 5'monophosphate
CSE	Cell surface engineering
CTL	Cytotoxic thymus lymphocyte
CTLA4	Cytotoxic T-lymphocyte protein-4
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate buffer saline
EC	Endothelial cell
ERK	Extracellular signal-related kinase
FBS	Fetal bovine serum
FMO	Fluorescence-minus-one
GNE	UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine
GSH	Glutathione
gtTGase	Guinea pig liver tissue transglutaminase
HABP1	Hyaluronan binding protein-1
HLA	Human leukocyte antigen
HMGB-1	High mobility group box-1
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IL-#	Interleukin-#
IgG	Immunoglobulin G

IRI	Ischemia reperfusion injury
ITAM	Immunotyrosine activating motif
ITIM	Immunotyrosine inhibitory motif
KIR	Killer immunoglobulin receptor
LPG-Sia	LPG-Q-Sia3Lac
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIC-A/B	MHC class I chain related protein-A or B
NFAT	Nuclear factor of activated T cells
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Neu	Neuraminidase (sialidase)
Neu5Ac	N-acetylneuramic acid
Neu5Gc	N-glyconeuramic acid
NK	Natural Killer
NKG2D	Natural killer group 2D
PBMC	Peripheral blood mononuclear cell
PDL-1	Programmed death ligand-1
PI3K	Phosphoinositide 3-kinase
PSGL-1	P-selectin glycoprotein ligand-1
PTEN	Phosphatase and tensin homolog
RPMI	Roswell Park Memorial Institute
Siglec-#	Sialic acid-binding immunoglobulin-like lectin-#
SHP-#	Src homology region 2 domain-containing phosphatase-#
sLe^x	Sialyl lewis-x
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
UW	University of Wisconsin
VCAM-1	Vascular cell adhesion molecule-1
WGA	Wheat germ agglutinin

Chapter 1. Introduction

1.1. Organ transplantation

The only therapeutic strategy for end stage organ failure is organ transplantation with the most common types being kidney, heart, liver, lungs, and pancreas. In Canada, 2, 936 transplants were performed in 2022 (Canadian Institute for Health Information 2023). Although this number is a great accomplishment, a major challenge lies in the incomplete longevity of transplanted organs. Most grafts eventually fail due to recognition of foreign molecules by the host's immune system resulting in rejection of the transplanted organ. Thus, a major limitation to the curative potential of this procedure is overcoming rejection of the graft. The response of the host's immune system as well as immune cells from the graft play critical roles in transplant rejection (Gan et al., 2022; Randolph et al., 2008). Inflammatory responses that form the early part of an immune response towards transplanted organs define how subsequent immune responses will be activated and how graft injury will occur. Understanding how the inflammatory response and subsequent rejection are controlled is important for developing therapeutics that prevent rejection.

Current therapeutic strategies to prevent transplant rejection involve the use of multiple broad acting immunosuppressive drugs to dampen the host immune response. Organ transplant patients receive multiple types of immunosuppressives at low doses to have a broad dampening affect on the host immune system while minimizing the risk of kidney damage. These drugs target the host's immune system non-specifically and are associated with many risks and complications (Roberts & Fishman, 2021; Tönshoff, 2020). Long term use of immunosuppressants is associated with increased risk of cardiovascular disease, bacterial infections from opportunistic pathogens, and cancer (Penn 1988; Dantal, Hourmant et al. 1998; Fishman and Rubin 1998; Lechler, Sykes et al., 2005). Despite the risks, the use of immunosuppressants have improved patient outcomes but longitudinal studies have shown that graft survival diminishes significantly overtime. The 10-year survival rates hover at ~50-70% for heart and liver transplants and just over 25% for lung and intestinal transplants (Giwa et al., 2017).

There is a need for new strategies to circumvent the use of immunosuppressants and for new methods to protect grafts from early damage to prevent transplant rejection. Promising areas of research focuses are on methods for nanoparticle delivery of immunosuppressants, ex-vivo cell transfer of tolerogenic immune cells, and organ engineering to convert foreign organs to match the recipient (Chow et al., 2019; Fisher et al., 2015; Juvet et al., 2014; Trzonkowski et al., 2009; B. Wang et al., 2021). Although encouraging, these strategies rely on complex and time-consuming strategies and have their own limitations and introduce new complications. For example, nanoparticle delivery of immunosuppressants and ex-vivo cell transfer of tolerogenic cells rely on consistent delivery of therapies to maintain immunosuppression and do not address the increased susceptibility to infections. Also, organ engineering requires decellularization of donor organs and recellularization with recipient stem cells which is a process that must occur far in advanced prior to transplantation. Therefore, strategies that address the use of immunosuppressants for immediate protection of donor grafts must be developed.

I have focused on a Cell Surface Engineering (CSE) approach to circumvent the use of immunosuppressants while reducing acute and chronic rejection. This strategy is employed during the transplantation process. The donated organ is perfused with a solution composed of a biocompatible and immunoregulatory polymer which coats blood vessels. In-vivo experiments using a mouse artery transplantation model and kidney allograft model demonstrated that CSE of the endothelial glycocalyx prevents acute and chronic rejection (Siren et al., 2021). However, the mechanism by which this occurs remains unknown.

Early inflammation as a trigger for transplant rejection

The immune response in transplantation is an interplay between the innate and adaptive immune responses. Mechanical and ischemic injury to the graft results in the release of damage associated molecular patterns (DAMPs) which generate a local proinflammatory environment (Roh & Sohn, 2018). These are endogenous molecules that are released from damaged or dying cells that bind to pattern recognition receptors, such as toll-like receptors (TLRs), on innate immune cells and leads to their activation. The initial innate immune response in transplant rejection consists of early activation of monocytes, neutrophils, and natural killer (NK) cells upon stimulation by DAMPs. For example, high mobility group box -1 (HMGB-1) is a protein localized in the nucleus of cells

and is released into the environment upon apoptosis. Extracellular HMGB-1 binds to TLR4 on innate immune cells to upregulate signaling pathways that cause nuclear localization of transcription factors to cause gene expression of pro-inflammatory cytokines. For NK cells, stimulation of TLRs can cause direct injury to graft cells by activation-induced cell death. Activation of innate immune cells results in the release of proinflammatory cytokines that contribute to the proinflammatory environment to enhance the ability for alloimmune recognition. Activation of antigen presenting cells (APCs) results in their expression of co-stimulatory molecules such as CD80/86 and presentation of donor antigens that leads to activation of T cells and the production of donor specific antibodies that causes transplant rejection (Randolph et al., 2008).

Early damage to the graft occurs during the organ procurement and transplantation processes, through ischemia reperfusion injury (IRI) where the sudden removal and subsequent restoration of blood flow into the donor graft causes direct cell death of ECs that line blood vessels (Cowled & Fitridge, 2011). Endothelial cells (ECs) are covered in a protective matrix, called the glycocalyx. The glycocalyx is made up of lipids, glycans and proteoglycans enriched in sugars, such as sialic acid, that protects ECs from damage by preventing accessibility to antigens and by displaying immune-inhibitory ligands (D'Addio et al., 2020; Ghosh, 2020; Varki et al., 2009). Injury results in shedding of the glycocalyx, which reduces its protective functions and is an early indicator of later transplant rejection (Mathis et al., 2021) (Figure 1.1). EC death causes the release of DAMPs such as HMGB-1 and heat shock proteins into the environment where they bind TLRs on monocytes, macrophages, and dendritic cells (DCs) (Roh & Sohn, 2018). The activation of TLRs causes expression of inflammatory cytokines to induce expression of cell adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on ECs (C. Y. Wang et al., 1998). Binding of leukocytes to these adhesion molecules causes the extravasation of immune cells across blood vessels and into transplanted organs. The infiltration of immune cells into the graft allows for alloimmune recognition by antigen presenting cells to generate an adaptive immune response to the graft. DCs are a link between innate and adaptive immune responses. In transplantation, inflammatory signals activate DCs to present alloantigens to T cells. DCs from the donor, which are present within transplanted organs, and DCs from the recipient both contribute to activation of alloreactive T cells (Randolph et al., 2008).

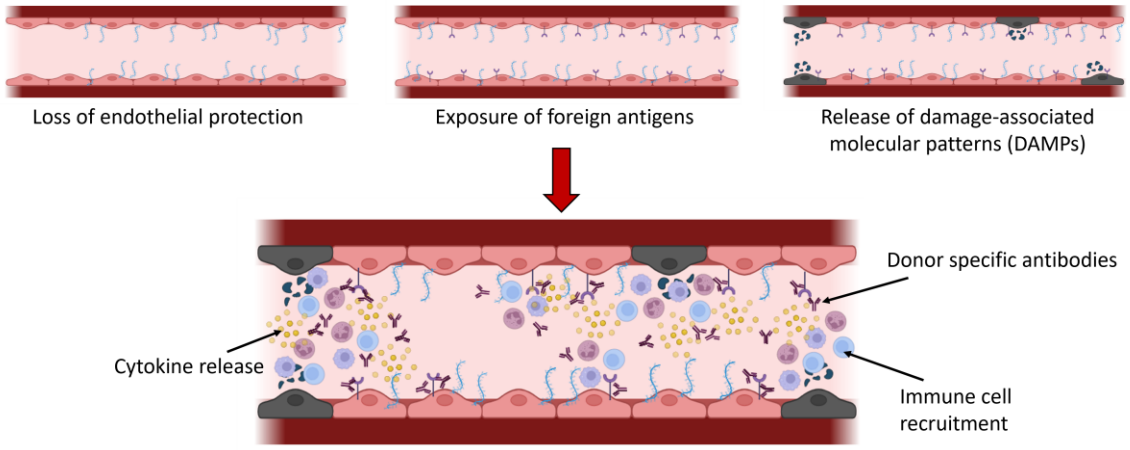


Figure 1.1. Loss of the endothelial glycocalyx contributes to early inflammation
Shedding of the glycocalyx causes ECs to become vulnerable to immune-mediated damage which contributes to the recruitment and activation of immune cells as well as the development of cytokines and donor specific antibodies. Made with Biorender™.

Direct and indirect alloimmune recognition leads to transplant rejection

APCs, such as DCs, macrophages, and monocytes, activate alloreactive T cells by recognizing donor derived foreign peptides that are presented on MHC molecules. These peptides are derived by injured ECs from the transplanted organ and are taken up by APCs, processed and then presented on MHC molecules (Alegre et al., 2016). Donor derived APCs carried by the transplanted organ and recipient APCs that infiltrate the graft activate recipient T cells resulting in alloimmune recognition. Whether T cells are activated by donor derived APCs or recipient APCs defines whether alloimmune recognition occurs directly or indirectly (Figure 1.3).

Direct alloimmune recognition involves donor derived APCs, that are carried by the transplanted organ, expressing foreign peptides on their MHC molecules. The mechanical and ischemic damage caused by the transplantation process causes donor APCs to become activated in response to blood vessel injury. Donor APCs upregulate the expression of foreign peptides as well as ligands that are essential for the activation of T cells ie. CD86 and CD40. They move from the graft and into recipient secondary lymphoid organs where they present foreign peptide on foreign MHC molecules to T cells that recognize the antigen on T cell receptors. T cells will also receive co-stimulatory signals through CD86 binding to CD28 and CD40 binding to CD40L. The resultant effect of successful peptide-MHC binding and co-stimulation causes activation of T cells leading to their expansion, differentiation into effectors, and infiltration to the graft. Activated cytotoxic

CD8 T cells recognize foreign peptides on ECs to directly kill them causing irreversible graft injury. Effector CD4 T cells secrete cytokines such as IFN-γ and TNF-α that compromise organ function.

Indirect alloimmune recognition involves the recognition of foreign peptides on self-MHC molecules. Foreign peptides are taken up by recipient APCs, processed, and presented on self-MHC molecules. Recipient leukocytes infiltrate the media and intima of blood vessels as part of the inflammatory process after transplantation. In this process they are exposed to DAMPs which bind to TLRs resulting in expression of co-stimulatory molecules CD80/86. APCs from the recipient phagocytose foreign peptides and display them on MHCI and MHCII molecules and move through the lymphatic system into lymph nodes and spleen to present foreign peptides on self-MHC molecules to T cells. The activation of CD4 T-helper type 1 cells in this way leads to the secretion of IFN-γ to cause graft cell dysfunction. (Tellides & Pober, 2007).

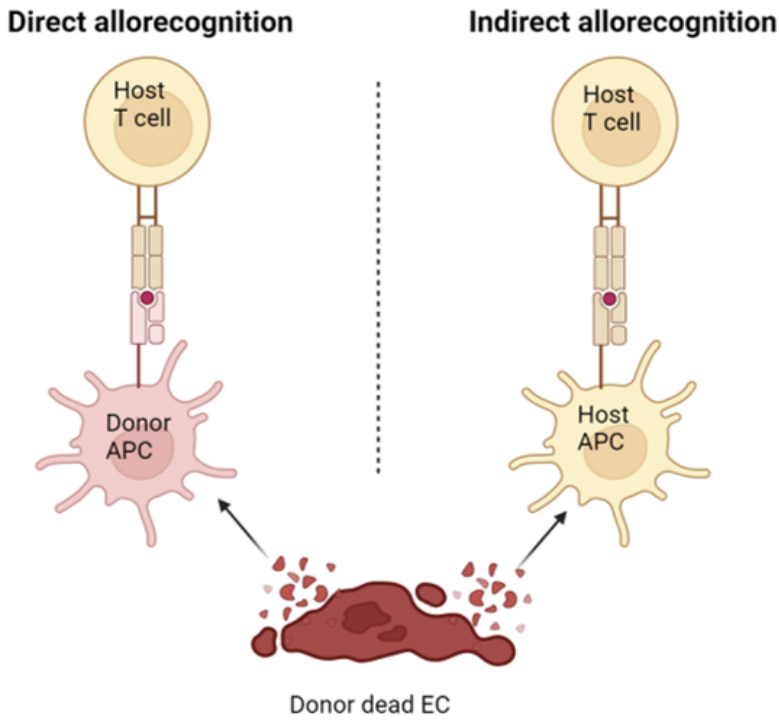


Figure 1.2. Mechanisms of allorecognition
Transplanted organs can be detected through direct and indirect allorecognition where antigens from donor dead endothelial cells (ECs) are presented to host T cells by antigen presenting cells (APCs). Made with Biorender™.

NK cells in transplantation

In addition to T cells that injure transplant blood vessels, NK cells also cause early vascular injury. NK cells can mediate cytotoxicity through two mechanisms: direct lysis by activation induced cell death and antibody-dependent cellular cytotoxicity (Pontrelli et al., 2020). NK cells are activated in transplanted organs through binding of activating ligands, such as MHC class I chain-related protein-A (MIC-A) and MIC-B that are induced on ECs upon injury and/or inflammation. Binding of MIC-A to activating killer immunoglobulin-like receptors (KIRs) such as natural killer group 2D (NKG2D) causes upregulation of cytotoxic pathways thereby causing direct lysis of ECs. NK cell activation can be inhibited by ligands such as self human leukocyte antigen-1 (HLA-1) molecules. Binding of HLA-1 molecules to inhibitory KIRs on NK cells deactivates these cytotoxic pathways thereby preventing EC death (Koenig et al., 2019). In the case of missing self HLA-1 molecules, which can occur when transplanted organs do not express the proper self HLA-1 molecules, NK cells are activated resulting in the direct lysis of ECs. Antibody-dependent cellular cytotoxicity is mediated by NK cells binding to donor reactive antibodies through activating FcγRIII receptors. These are two mechanisms by which NK cells can cause cytotoxicity to transplanted organs. Genetic analysis of biopsies of renal transplant recipients demonstrated that mismatched HLA-1 molecules can explain microvascular lesions caused by direct injury by NK cells (Koenig et al., 2019). The presence of other activating ligands, such as MIC-A/B, have been reported in biopsies of patients undergoing acute and chronic allograft rejection. Also NKG2D expression was found in urine samples of patients undergoing acute kidney allograft rejection (Ascon et al., 2006). These results indicate a role for NK cells in transplantation where NKG2D could be a biomarker for acute allograft rejection.

1.2. The endothelial glycocalyx and sialic acid

Architecture of blood vessels

Healthy arteries have three distinctive layers: intima, media, and adventitia (Figure 1.2). The adventitia is the outermost layer and is rich in collagen, fibroblasts, and perivascular nerves. It functions as a dynamic layer allowing for cell trafficking, growth and repair of the vessel, and mediates communication with smooth muscle cells in the media and ECs in the intima (Gutterman, 1999; Haurani & Pagano, 2007; Sartore et al., 2001).

It is also a source of micro vessels that form a conduit for immune cell infiltration. As mentioned, the media is comprised of smooth muscle cells interlined with elastic laminae responsible for vascular constriction and dilation (Havelka & Kibbe, 2011). The intima is the space between the ECs that line the lumen of vessels and the media. Blood vessels are the first part of contact between the organ transplant recipient and the donor organ. Injury of transplant arteries is a feature of severe acute transplant rejection. In healthy arteries the intima is thin and it becomes thickened during many vascular diseases which blocks the blood flow through affected arteries (Herbert & Stainier, 2011).

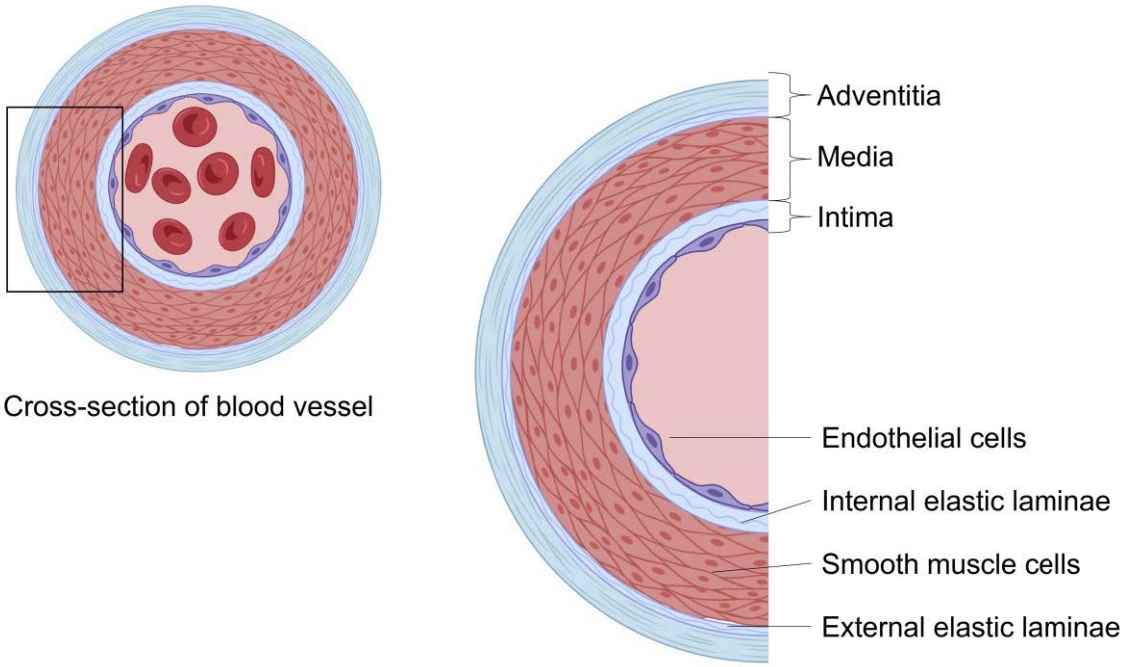


Figure 1.3. Schematic representation of essential components of blood vessels
Blood vessels are comprised of three layers: intima, media, and adventitia. Each layer serves multiple functions to maintain the health and proper functioning of blood vessels. Damage to any of these components results in the disruption of vasomotor functions. Made with Biorender™.

Acute and chronic allograft rejection causes changes in the organization of blood vessel composition resulting in vascular injury and dysfunction. Acute rejection is mediated by direct vascular injury. ECs expressing major histocompatibility complex (MHC) molecules present foreign peptides to cytotoxic alloreactive memory cluster of differentiation-8 (CD8) T cells which directly target foreign ECs for death. In addition, acute antibody-mediated rejection is caused by circulating immunoglobulin G (IgG) antibodies that result in vascular injury upon Fc fragment binding to cytotoxic immune cells that have Fc receptors. During acute rejection, leukocytes damage ECs by releasing cytotoxic

granules causing EC death. Leukocytes infiltrate into the vascular tissue, resulting in damage and loss of smooth muscle cells as well as the integrity of elastic laminae. This causes an increase in the healing process resulting in fibrinoid necrosis where irreversible damage occurs due to antigen-antibody complexes mixed with fibrin in vessel walls (Abrahimi et al., 2015). Chronic rejection involves intimal thickening that leads to concentric narrowing of the arterial lumen which reduces blood flow through the lumen and leads to downstream ischemia of affected grafts. T cell infiltration and activation within vessels results in pro-longed secretion of IFN-y. IFN-y causes uncontrolled proliferation of smooth muscle cells, causing reorganization of vessel components and progressive movement of smooth muscle cells into the intima. (Geraghty et al., 1996).

The glycocalyx matrix

The endothelial glycocalyx is a coating of carbohydrates, proteins, and glycoproteins found on the surface of ECs and was first described as an essential part of the “hematoparenchymal barrier” (Zweifach, 1955) (Figure 1.4). The glycocalyx extends 50-100nm into the lumen and has filamentous spacing of 20nm which is indicative of its filtration capabilities of small molecules. Proteins such as collagen and elastin provide structural and elastic support to the glycocalyx (Plopper et al., 2007). Glycoproteins are a large family of membrane-bound proteins that have sugars, or glycans, covalently linked to an amino acid scaffold. Typical glycoproteins have glycosidic linkages to short highly branched glycan chains with no repeating units and are capped with sialic acid residues. Glycosylphosphatidylinositol (GPI)-anchored glycoproteins and proteoglycans are subclasses of glycoproteins found on the glycocalyx. GPI-anchored proteins are linked to membranes by mannose sugars and an inositol residue and function as enzymes and receptors (Paulick & Bertozzi, 2008). In contrast to typical glycoproteins, proteoglycans have long unbranched glycan chains with repeating disaccharide units that are covalently bound through a tetrasaccharide bridge. These are heavily sulfated heparan and chondroitin chains which confer a negative charge that function as an anionic site to mediate ion exchange by attracting sodium, potassium, and calcium ions. In addition, due to their strong structural and hydrophilic nature, proteoglycans can aggregate to form a cushion that can withstand compressional stress (Frevert & Sannes, 2005). Hyaluronan are long glycosaminoglycan chains that are non-membrane bound and are highly hydrophilic. They can bind up to 1000 times its weight in water molecules to form a spatial buffer which can block high molecular weight proteins and allow diffusion of ions and small

molecules through the glycocalyx (Garantziotis & Savani, 2019). Embedded on the mesh of proteoglycans and glycoproteins are soluble components, such as albumin and orosomucoid, derived from ECs or the blood stream. The interaction between soluble proteins, proteoglycans, proteins, and glycoproteins provides strength and stability for the appropriate functioning of the glycocalyx.

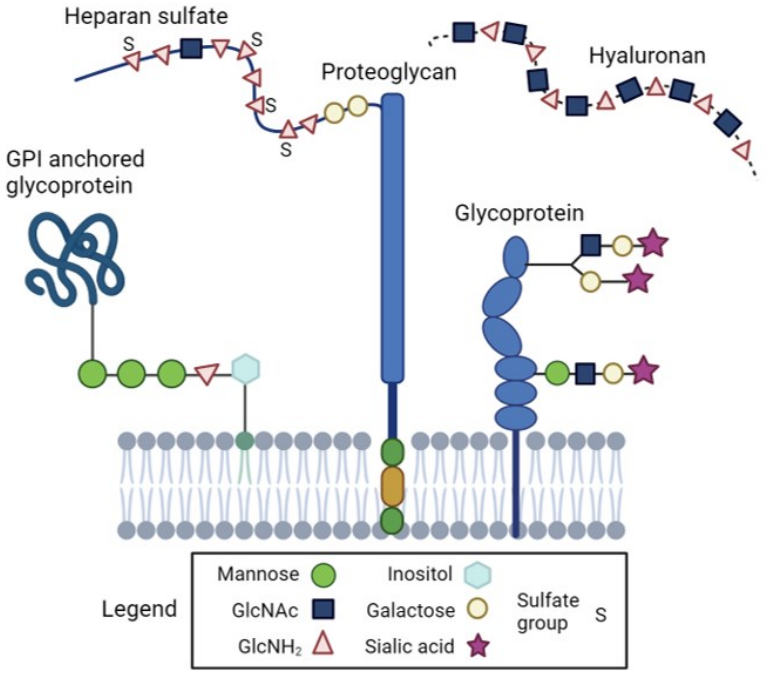


Figure 1.4. Glycan components of the endothelial glycocalyx
The glycocalyx is abundant in glycans such as mannose, galactose, N-acetylglucosamine (GlcNAc), d-glucosamine ($GlcNH_2$), and inositol residues. Sialic acids and sulfate groups on glycan chains confer the overall negative charge of the glycocalyx. Made with Biorender™

The individual functions of proteins, glycoproteins, proteoglycans, and glycan chains on the glycocalyx encompass the multi-functional properties of the glycocalyx. An essential function is to serve as a physical barrier that protects ECs from injury. The overall negative charge and bulkiness of the matrix limits the access of macromolecules to ECs. Damage to the endothelial glycocalyx increases vascular permeability in mouse models with even partial enzymatic removal of matrix components. In mouse models of lipopolysaccharide-induced lung injury, exacerbation of glycocalyx injury with heparinase treatment resulted in significantly increased infiltration of leukocytes into lung tissue (Masola et al., 2022). These findings demonstrate the barrier functionality of the glycocalyx. Other functions involve its capability to mediate communication between blood

vessel-vessel wall interactions and its ability to sense sheer stress. Injury of the glycocalyx causes the loss of its protective functions and increases vascular permeability both of which perturb vascular homeostasis. Recent studies have described shedding of the glycocalyx as a biomarker for acute allograft rejection (Inkinen et al., 2019; Schiefer et al., 2015; Sladden et al., 2019).

Sialic acid structure and biosynthesis

Sialic acids belong to a family of negatively charged acidic monosaccharides (Yanhong Li & Chen, 2012; Varki et al., 2009). This sugar consists of a five-carbon cyclic ring with a three-carbon exocyclic side chain. Sialic acids differ in their substituents at C5 and in their glycosidic linkages. For example, N-acetyl neuraminic acid (Neu5Ac) has an acetylated amino group, whereas N-glycolylneuraminic acid (Neu5Gc) has a glycosylated amino group (Figure 1.5). The glycosidic linkages at the C2 of sialic acid alters the steric configuration of the molecule. The difference in their steric configuration determines the ability of the molecules to bend and rotate which contributes to their ability to bind to other molecules and different receptors.

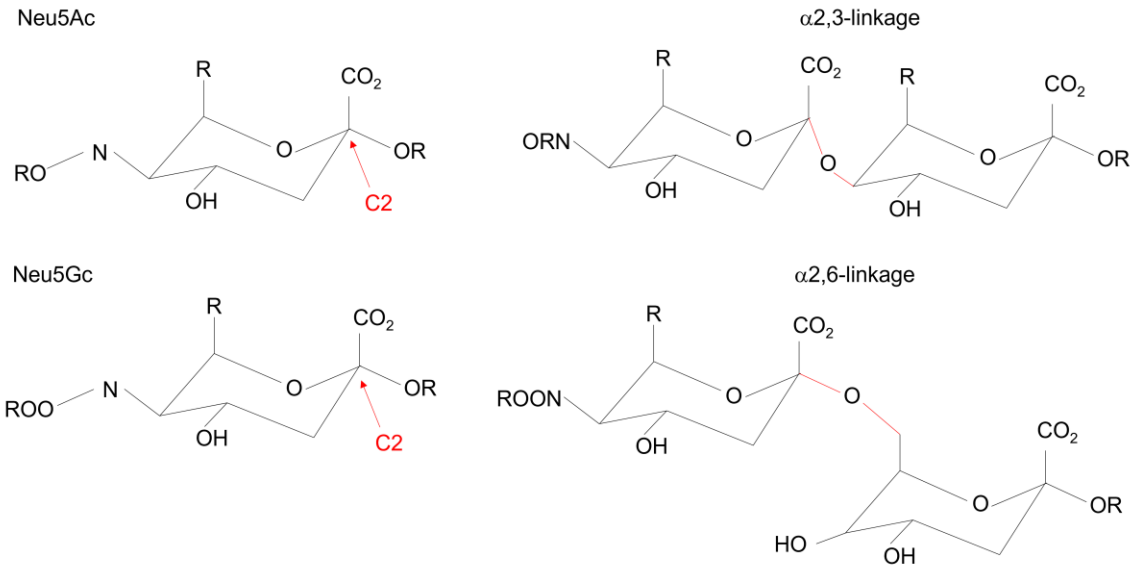


Figure 1.5. Glycosidic linkages affect sialic acid presentation
The binding of different steric configurations of sialic acids Neu5Ac and Neu5Gc is caused by the type of glycosidic linkage at the C2 carbon which can introduce kinks in the presentation of sialic acid(Yanhong Li & Chen, 2012; Varki et al., 2009).

The synthesis of sialic acid relies on the coordination of multiple enzymes for the formation of sialyl glycoconjugates (Yanhong Li & Chen, 2012). An intricate conversion of

the initial substrate, glucose, to a Neu5Ac precursor is achieved in the cytosol. A crucial step in the synthesis of sialyl glycocongugates is the formation of ManAc from UDP-GlcNAc. This step is an epimerization catalyzed by a kinase in its tetrameric form encoded by the gene UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine (GNE). Once the final precursor is made, free sialic acid combines with an activated donor in the nucleus, cytidine 5'monophosphate (CMP), to form CMP-Sia. The formation of CMP-Sia is the rate limiting step of protein sialylation and the enzyme responsible for this reaction is CMP-Sia synthetase. CMP-Sia is shuttled from the nucleus to the Golgi apparatus where sialyation of glycoconjugates occurs. A family of 20 sialyltransferases is responsible for the transfer of sialic acid from CMP to glycoconjugates. Each sialyltransferase transfers a single sialic acid molecule to a specific sugar substrate. Regulation of sialic acid occurs in two ways: inhibition of its biosynthesis by negatively regulating GNE kinase and removal of sialic acid on glycoconjugates by sialidases. When concentrations of CMP-Sia in the cytosol are high, GNE kinase forms a dimer instead of a tetramer which prevents the kinase from epimerizing UDP-GlcNAc to form ManAc. As well, sialidases, also known as neuraminidases (NEU), are produced by mammalian cells and remove sialic acid on glycoconjugates to inhibit its bioactivity. There are four types of sialidases in mammals that differ in substrate specificity and cellular localization. NEU1 is the most common type of sialidase found in mammals and hydrolyzes the glycosidic linkages of Sia. Differences in expression of Sia on the surface of cells contributes to the differences in cell binding and cell signaling between immune cells and ECs.

Sialic acid expression and function

Sialic acid expression is widespread throughout the human body including in cells of the brain, immune system, epithelial lining, and blood vessels (Ghosh, 2020). The expression of sialic acid, its spatial organization, and its substituents play a significant role in their function.

A critical component of the immune response is leukocyte extravasation from the lumen to the target tissue (D'Addio et al., 2020). Activated ECs express E-selectin to mediate the loose tethering of immune cells to the EC membrane by binding to P-selectin glycoprotein-ligand 1 (PSGL-1) on leukocytes. PSGL-1 is a heavily O-glycosylated molecule with a capped sialyl Lewis-x (sLe^x) and has tyrosine sulfate residues. A critical component of PSGL-1 is the sLe^x that has sialic acid which allows for binding to E-selectin

to occur. The binding of PSGL-1 to E-selectin mediates the “rolling” mechanism of leukocyte migration and is the first step in leukocyte extravasation. The expression of sialic acid is also critical for immune cell signaling. Sialic acids can participate directly in immune cell signalling by binding to sialic acid-binding immunoglobulin-type lectins (siglecs) (Crocker et al., 2007).

1.3. Siglecs

Siglecs are sialic acid binding receptors that are expressed on the plasma membrane of mouse and human immune cells (Crocker et al., 2007). The human family of siglecs contains over 10 members, whereas the mouse family of siglecs contains 9 members. Mouse and human siglecs have paralogs that allow translation of findings in murine models to human biology, although there are also important species differences. For instance, humans have 9 siglec-3 (CD33) related siglecs, whereas mice have 5 CD33-related siglecs. The structure and expression of siglecs relate to their function.

The structure of siglecs

Siglecs are membrane proteins with extracellular, transmembrane, and intracellular domains (Crocker et al., 2007). The extracellular domain contains a variable region (v-set) and a constant region (c2-set). Both v-set and c2-set regions contain immunoglobulin domains, with the v-set region mediating sialic acid binding. The v-set region contains a conserved positively charged arginine residue which binds to its negatively charged sialic acid. Variation of the polypeptide sequence in the v-set region determines the amount of exposure arginine has to its ligands which results in different siglecs having preferential binding to different types of sialic acids. For example, siglec-2 (CD22) has preferential binding to α2,6 sialic acids whereas siglec-7 prefers α2,8 (Erickson et al., 1996). The transmembrane region is a scaffold that allows for the propagation of signals upon siglec engagement. The intracellular region of siglecs differ from each other in their expression of immunoreceptor tyrosine motifs. Some siglecs express immunoreceptor tyrosine inhibitory motifs (ITIMs), whereas others express immunoreceptor tyrosine activating motifs (ITAMs) or both. The tyrosine molecules within the ITIMs and ITAMs can become phosphorylated to become a docking site for tyrosine binding molecules. Upon siglec engagement, these motifs recruit adaptor molecules that propagate the signal to upregulate or downregulate immune cell activity.

Immune cell expression of siglecs

Different immune cells express different siglecs which contribute to the cell type specific regulation of their effector functions (Table 1.1). Siglecs-1 and 2 are conserved siglecs. Siglec-1, also known as sialoadhesin, is the most abundantly expressed siglec on macrophages (Hartnell et al., 2001). Siglec-1 does not have an intracellular domain and mediates adhesion as well as clathrin-dependent endocytosis of viruses upon binding to sialic acid. CD22 is a B cell restricted siglec that regulates BCR signaling (Doody et al., 1995; Erickson et al., 1996; Kelm et al., 1994). All other siglecs are CD33-related isoforms and are expressed on myeloid cells such as monocytes and DCs (Garnache-Ottou et al., 2005). Interestingly, CD33 becomes aberrantly expressed on T cells stimulated with anti-CD3 and IL-2 on B cells in patients with Behcet's disease, which makes it a target for antibody therapies (Eksioglu-Demiralp et al., 1999; Nakamura et al., 1994) .

Table 1.1. Siglec expression on human and mouse immune cells

	Human siglec expression						
Siglec	Sialic acid linkage	ITIM?	Monocytes / DCs/ macrophages	Neutrophils	T cells	NK cells	B cells
Siglec-1	α2,3	No	Macrophages/DCs	-	-	-	-
Siglec-2	α2,6	Yes	-	-	-	-	+
Siglec-3	α2,6	Yes	Monocytes/ macrophages	-	-	-	-
Siglec-5	α2,6	Yes	Monocytes	+	-	-	-
Siglec-7	α2,3 α2,8	Yes	Monocytes/DCs	-	+	+	-
Siglec-9	α2,3	Yes	Monocytes/DCs	+	+	+	-
	Murine siglec expression						
Siglec-1	α2,3	No	Macrophages	-	-	-	-
Siglec-2	α2,6	Yes	-	-	-	-	+
Siglec-3	α2,6	Yes	Macrophages	+	-	-	-
Siglec-E	α2,3	Yes	Monocytes/DCs/ macrophages	+	+	+	-
Siglec-G	α2,3	Yes	-	-	-	-	+

Based on their immunoregulatory properties, siglecs have been identified as potential immune checkpoint molecules (Duan & Paulson, 2020). Immune checkpoint molecules are molecules that inhibit immune activity to 'decide' whether to activate or inactivate immune cells. T cells found within the tumor microenvironment express siglecs-7 and 9 (Ibarlucea-Benitez et al., 2021; Jandus et al., 2014). Since tumor cells are known

to express sialic acid, the murine homolog of siglec-9, siglec-E, was tested in-vitro to determine siglec-E binding. After identifying siglec-E expressing T cells within the mouse tumor microenvironment, experiments were performed with siglec-E knock out mice. The results demonstrated that lack of siglec-E reduces tumor burden (Ibarlucea-Benitez et al., 2021). This study did not only identify siglec-7 and 9 as a new therapeutic target, but also it confirmed the inhibitory role of siglecs in preventing immune cell activation. Understanding the mechanism by which siglecs upregulate or downregulate immune cell activity is essential in the development of therapeutics.

Signaling pathway of immune inhibitory siglecs

Sialic acid binding to immune inhibitory siglecs causes downstream deactivation of pro-inflammatory pathways in immune cells (Figure 1.6). These pathways involve adaptor proteins and enzymes to propagate inflammatory signals causing, cell proliferation, activation, differentiation, and gene expression of proinflammatory cytokines: TNF-α, IL-1, IL-2, and IL-6. The protein kinase B/Akt pathway involves the activation of phosphoinositide 3-kinases (PI3Ks) which phosphorylates the inhibitory KB kinase (IKK) that is complexed with nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB). This causes nuclear translocation of NFKB resulting in proinflammatory gene expression (Liu et al., 2017). The mitogen activating protein kinase (MAPK) pathway involves a series of MAPKs including extracellular signal-related kinase (ERK) which also participates in the nuclear translocation of NFKB (Seo et al., 2013). Upon sialic acid binding to inhibitory siglecs, tyrosine residues in ITIMs self-phosphorylate forming a docking site for src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHP-2. Activation of these phosphatases cause dephosphorylation essential proteins such as ERK thereby downregulating the Akt and MAPK pathways (J. Cai et al., 2006; Lu et al., 2003).

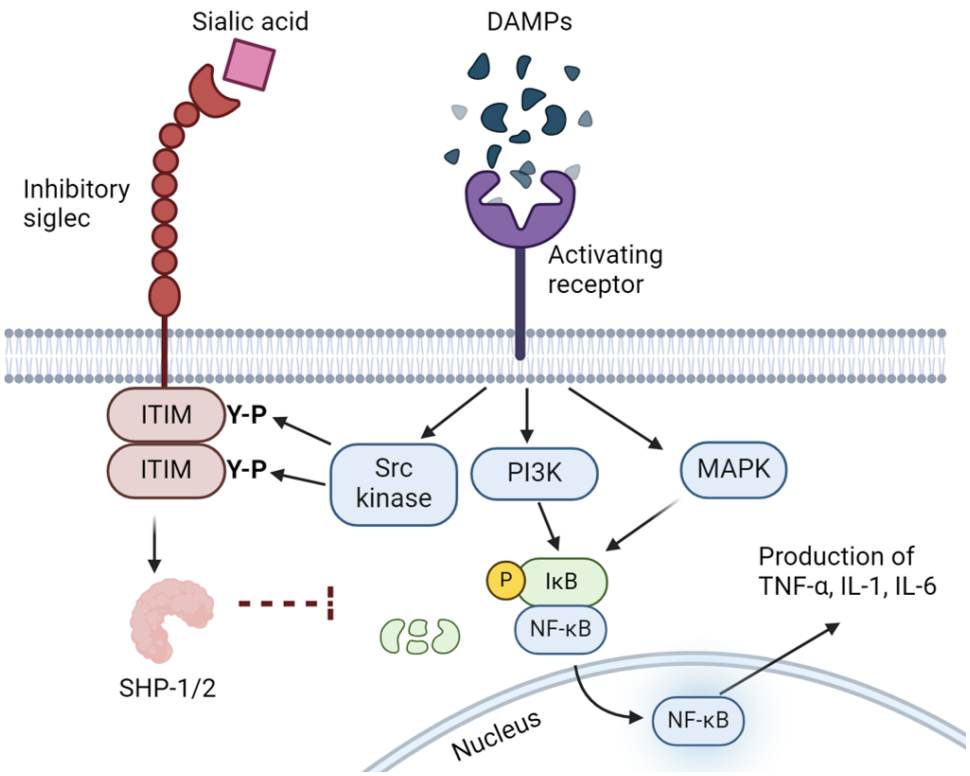


Figure 1.6. Immune inhibitory siglecs deactivate inflammatory pathways

Binding of sialic acid to inhibitory siglecs on immune cells causes ITIM motifs to self-phosphorylate their tyrosine residues to recruit and activate SHP-1 and SHP-2. Activation of SHP-1/2 causes dephosphorylation of key proteins and indirectly prevents nuclear translocation of NFKB, as indicated with the dotted line, thereby disrupting pro-inflammatory gene expression. Made with BioRender™.

Cis and trans signaling of sialic acid through siglecs

Binding of sialic acid to siglecs occurs in cis or trans to modulate immune cell activity (Crocker et al., 2007). Cis interactions occur when sialic acid expressed on a cell binds to siglecs on the same cell. The abundance of sialic acid on a cell allows for cis interactions which can upregulate or downregulate their activity. For example, siglec 2 or CD22, modulates B cell activation upon B cell receptor (BCR) engagement (Khatua et al., 2013; Macauley & Paulson, 2014; Nitschke et al., 1997). In this scenario, abundance of sialic acid on B cells act as a continual source of CD22 ligand that activates its receptor once it is induced. When BCRs are engaged, sialic acids on B cells bind to CD22 and inhibit BCR signaling. The significance of cis interactions on B cells relates to the signalling threshold of BCRs. When CD22 -/- mice are stimulated with a B cell mitogen, B cells are quickly activated and die earlier. The lack of CD22 reduces the signaling threshold of B

cells and causes premature B cell death due to overstimulation. Trans signalling occurs when siglecs bind to sialic acid on other cells. For example, when mouse lung tissue is stimulated with an allergen, siglec-F on eosinophils bind to the α2,3 sialic acid found on epithelial cells. Clinical studies have shown that asthmatic patients have a high level of eosinophils expressing siglec-8, a human paralog of siglec-F, from bronchoalveolar lavage fluid experiments. Since inflammation in the lung is associated with increased levels of eosinophils, siglec-8 is expected to play a role in the pathogenesis of asthma (Kiwamoto et al., 2013).

1.4. Current strategies to prevent allograft rejection

Broad-acting immunosuppressants

The most common types of therapies used to help prevent transplant rejection are immunosuppressive drugs (Tönshoff, 2020). These drugs non-specifically inhibit T cell activation, which prevents rejection and has allowed for wide-spread success of organ transplantation. Cyclosporine and tacrolimus are the most common immunosuppressant drugs and both act by inhibiting (Matsuda & Koyasu, 2000). In T cells, calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT), a transcription factor, which allows NFAT to translocate to the nucleus and drive many aspects of T cell activation, including the production of IL-2. Cyclosporine and tacrolimus act similarly to inhibit calcineurin from dephosphorylating NFAT thereby preventing T cell activation. Although these drugs are associated with decreased transplant rejection rates, they can both cause nephrotoxicity, hypertension, neurotoxicity, and other adverse effects in addition to increased susceptibility to infections (Tönshoff, 2020).

Another immunosuppressive strategy involves co-stimulatory blockade of the CD80/86 molecules on DCs with CD28 on T cells (Ding et al., 2021). Because T cell activation requires co-stimulation through CD80/86 molecules, blocking this feature of activated DCs can prevent T cell activation and inhibits kidney transplant rejection. Cytotoxic T-lymphocyte associated protein 4 (CTLA4) is a protein expressed by effector T cells late after activation as well as on T regs. It has a much higher affinity to CD80/86 molecules than CD28 but does not induce signal transduction. In this way, the engagement of CTLA4 with CD80/86 prevents co-stimulatory CD86 binding to CD28 and ‘turns off’ T cell co-stimulation. As such, CTLA4IgG is chimeric protein that prevents co-

stimulation of T cells in transplant recipients. It is comparable in its ability to prevent kidney transplant failure when compared with calcineurin inhibitors. Importantly, its effectiveness highlights the central role of early inflammatory activation of innate immune cells, such as dendritic cells, in the long-term inhibition of transplant rejection.

Cell Surface Engineering for the prevention of allograft rejection

A novel CSE strategy involves an adjustment to the organ procurement protocol which protects the graft from ischemic and mechanical injury during the transplantation processes (Siren et al., 2021). As discussed, early damage to transplants causes shedding of the endothelial glycocalyx that provides protection to blood vessels. This damage is an early trigger for inflammation and immune activation. A CSE approach was developed to preserve the protective functions of the glycocalyx during the transplantation process by modifying the vascular endothelium with a bioactive polymer termed, LPG-Sia. This is achieved by modifying graft vascular cells during the organ procurement process using an enzyme, guinea pig transglutaminase (gtGase), to covalently conjugate bioactive polymers to the glycocalyx of ECs (Figure 1.7).

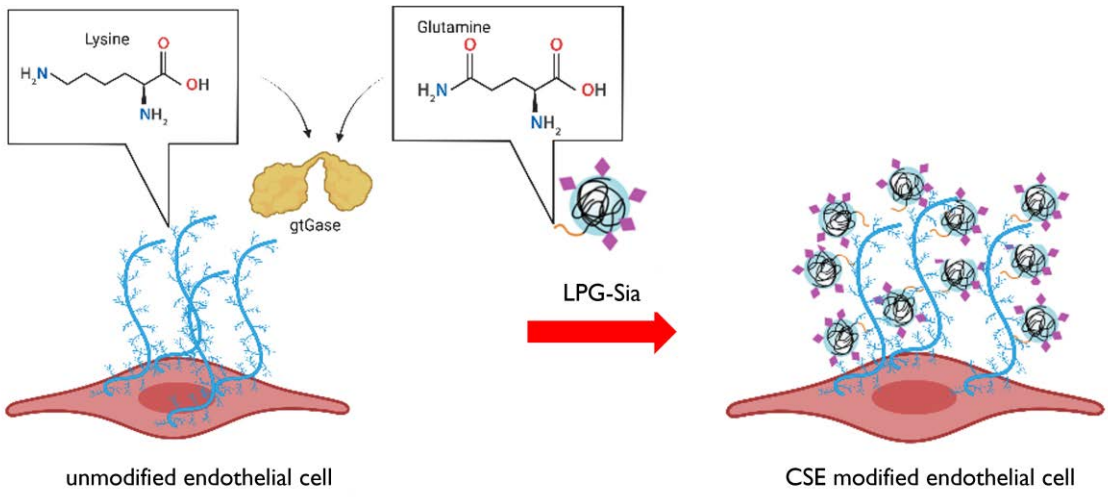


Figure 1.7 CSE modification of ECs with LPG-Sia
CSE modification of ECs involves gtGase enzyme forming an amide bond from glutamine residues on LPG-Sia to lysine residues on ECs. This irreversible covalent addition of LPG-Sia to ECs allows for the presentation of Sia residues towards the lumen to allow for immune cell regulation.

LPG-Sia is an α-2,3 linked sialic acid-containing polymer and when added to transplant blood vessels, it inhibits artery and kidney transplant rejection. Previous experiments demonstrated that LPG-Sia prevents immune targeting partly by sterically hindering leukocyte adhesion. When examined in preclinical models of vascular and

kidney rejection, CSE modification with LPG-Sia substantially reduced acute and chronic rejection. These results indicate that CSE modification with LPG-Sia may be applied to organ transplants before implantation. This could circumvent or reduce the use of immunosuppressants while improving the quality of the donated organ. Therefore, understanding the mechanisms by which CSE modification of ECs with LPG-Sia prevents immune-mediated allograft rejection is important. As discussed earlier, sialic acid has immunoregulatory properties and protective functions. This thesis provides preliminary evidence on how LPG-Sia inhibits immune cell activity. As well, a mechanism by which LPG-Sia could downregulate immune cell signalling is proposed.

Chapter 2. LPG-Sia inhibits innate immune cell mediated cytotoxicity of ECs

2.1. Introduction

During the organ transplantation process, mechanical and ischemic damage triggers the shedding of the protective endothelial glycocalyx. The glycocalyx is a matrix of proteoglycans and glycosaminoglycans and is rich in sialic acid. It is a physical barrier between ECs and the lumen, it senses shear stress, and is a scaffold for immune cell-EC signaling. Therefore, loss of the glycocalyx results in loss of its protective functions increasing vulnerability of ECs towards immune mediated injury (Sladden et al., 2019).

Immune cells transition from an inactive to an active state after being stimulated by DAMPs, such as HMBG-1, that are released by dying ECs. DAMPs bind to TLRs on monocytes and macrophages, which secrete tumor necrosis factor-α (TNF-α), IL-6, and IL-8 to direct the inflammatory cascade (Rao et al., 2007). Induction of TNF-α upregulates the expression of key cellular adhesion molecules, ICAM-1 and VCAM-1, that increases leukocyte adhesion to ECs (D'alessio et al., 1998). Tight binding of leukocytes to ECs mediates the leukocyte migration into the donor graft and leukocyte targeting of graft cells for killing. In response to inflammatory signals, activated NK cells increase expression of KIRs, such as NKG2D. NKG2D binds to MIC-A and MIC-B which is an NK cell activating ligand that is increased in expression on injured or inflamed ECs. Binding of NKG2D to their ligands causes degranulation of cytotoxic granules which can be visualized by externalization of CD107a on NK cells. This causes target cell lysis through induction of cell death by granzyme and perforin. Killing of ECs by cytotoxic T lymphocytes and NK cells results in loss of ECs and irreversible vascular injury (Pontrelli et al., 2020).

To prevent immune-mediated vascular injury, it is essential to protect the graft during the organ procurement process. Ischemic injury causes degradation of the endothelial glycocalyx, which enables the adhesion and recruitment of immune cells (Cancel et al., 2016). The CSE protocol modifies ECs with a bio-compatible and sialic acid containing polymer, LPG-Sia, which has shown to prevent acute and chronic rejection in mouse artery transplants (Siren et al., 2021). In-vitro results showed that LPG-Sia was protective to ECs with either an α2,3-linked or α2,6-linked sialic acid. Since innate immune

cells such as monocytes, DCs and NK cells bind to α2,3-linked sialic acid preferentially through siglec immunoregulatory receptors, all future experiments were performed with α2,3-linked sialic acid LPG-Sia. Therefore, I hypothesized that sialic acid on LPG-Sia protects ECs and can downregulate immune cell cytotoxicity towards ECs. To determine the affects of immune cell cytotoxicity towards cell surface modified ECs, I developed and used an in-vitro co-culture assay that involves co-culture of ECs with cytokine-stimulated peripheral blood mononuclear cells (PBMCs). I found that loss of endogenous sialic acid on ECs significantly increases immune-mediated cytotoxicity towards ECs. CSE modification of ECs with LPG-Sia protects ECs from immune-mediated EC death from PBMCs and NK cells. To understand whether immune cells are deactivated by LPG-Sia, I performed preliminary assays to neutralize candidate receptors on immune cells that could bind to sialic acid and deactivate them. Candidate receptors, siglec-7 and siglec-9, are expressed on NK cells and T cells (Crocker et al., 2007). These siglecs downregulate immune cell activity through ITIMs to activate phosphatase SHP-1 (Blasioli et al., 1999). SHP-1 dephosporylates proteins in the Akt and MAPK pathways to prevent nuclear translocation of NFKB resulting in deactivation of inflammatory pathways (J. Cai et al., 2006; Lu et al., 2003). I found that both siglec-7 and siglec-9 were required to deactivate PBMCs from targeting ECs for death.

2.2. Materials and methods

Human endothelial cell culture

EC lines were cultured in T-75 flasks at 37°C and 5% CO_2 up to 70% confluency and passaged using 0.25% trypsin and 0.05% EDTA. EAhy.926 cells (CRL-2922; American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified eagle medium (DMEM) (Thermofisher, Waltham, MA) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Burlington, MA) up to 50 passages. Human umbilical vein endothelial cells (HUVECs) (CC-2517 (single donor) Lonza) were cultured using endothelial basal medium (M200500; Thermofisher, Waltham, MA) and large vessel endothelial supplement (A1460801; Thermofisher, Waltham, MA) up to 10 passages.

Human peripheral blood isolation and culture

PBMCs were cultured in Roswell Park Memorial Institute (RPMI) with 10% FBS at 37°C and 5% CO2 overnight and were stimulated with 1000 IU/ml recombinant human IL-

2 (200-02; Peprotech, Rocky Hill, NJ). Stimulated PBMCs were washed and resuspended at 3 million cells/ml with RPMI and 10% FBS and subjected to co-culture assays.

NK cell isolation from PBMCs

NK cells were isolated using the EasySep^{TM} human NK cell isolation kit (17955; STEMCELL Technologies, Vancouver, BC). Briefly, PBMCs were resuspended in Dulbecco's phosphate buffer saline (DPBS) with 2% FBS and mixed with a cocktail of magnetic beads to negatively select NK cells. NK cells in the supernatant were collected by centrifugation at 400g for 5 minutes, then subjected to further analysis.

Sialidase modification of ECs

After culturing a monolayer of ECs, either EAhy.926 or HUVEC, sialidase, also known as neuraminidase, was used to remove sialic acid in cytotoxicity assays. Sialidase from *Clostridium perfringens* (11585886001; Sigma-Aldrich) or *Micromonospora viridifaciens* (provided by Dr. Andrew Benett's laboratory) was used at 0.05ug/ml in DMEM. ECs were washed twice with DMEM, then incubated with sialidase for 1 hour at room temperature in static conditions. After washing cells with DMEM, ensuring the removal of sialidase, endothelial cells were subjected to co-culture experiments.

CSE modification of ECs

ECs at 70% confluency were trypsinized and plated in 96-well flat bottom plates (83.3924; Sarstedt, Numbrecht, Germany) at a concentration of 10,000 cells/well. After cells reached 100% confluency, cells continued to grow for another 4 days at 37°C and 5% CO_2. Cells were washed with ice cold University of Wisconsin (UW) solution twice and incubated with UW solution supplemented with 3mM glutathione (GSH), 5mM calcium chloride ($CaCl_2$), 0.2 Uml^{-1} gtTGase and 0.5mM LPG-Q-Sia3Lac (LPG-Sia). Cells were incubated at 4°C for 30 minutes at static conditions. Cells were washed twice with ice cold DPBS (14190250; Thermofisher, Waltham, MA) then subjected to further analysis.

PBMC cytotoxicity assay

EAhy.926 ECs were seeded at 1, 000 cells/well on TC treated 96-well flat bottom plates in DMEM + 10% heat-inactivated FBS. Cells should form a 100% confluent monolayer after 4 days without media change with ~10 000 cells/well. After 7 days of culturing, cells were washed with serum-free DMEM twice and incubated with 2uM of

DMEM with CellTracker Green™ at 37°C and 5%CO_2 for 20 minutes. Cells were washed with ice cold PBS and underwent CSE modification or sialidase modification as described earlier in triplicates. Modified or unmodified ECs were co-cultured with PBMCs at a concentration of 10:1 PBMC to EC ratio. PBMC and EC co-culture was incubated for 20 hours at 37°C and 5%CO_2. Co-culture plates were centrifuged at 300g for 10 minutes and the supernatant was collected and transferred to another transparent 96-well flat bottom plate. Fluorescence in the supernatant was measured at 485/530λ with extended dynamic range and PBS only control wells using a Tecan Infinite M200Pro fluorometer. Final measurements of EC death were calculated after subtracting background fluorescence of ECs with CellTracker Green™. After 3 experimental replicates were performed, fold-change in each experiment was calculated using the following equation:

$$\frac{average\ fluorescence\ from\ 3\ technical\ replicates}{average\ fluorescence\ in\ unstimulated\ PBMC\ and\ EC\ control} = fold\ change$$

Flow cytometry

Flow cytometry analysis was performed on unmodified and modified EAhy.926 cells to measure the amount of sialic acid using wheat germ agglutinin. EAhy.926 cells were grown for 7 days in a 96 well flat-bottom plate and modified with viral sialidase or with LPG-Sia using the CSE protocol. Cells were washed twice with DPBS and stained with FITC-conjugated wheat germ agglutinin at 2uM in PBS at room temperature in static conditions in the dark. Cells were washed and resuspended in DPBS + 2% FBS for flow cytometric analysis.

NK cells from cytotoxicity experiments were collected by centrifuging the supernatant in ice cold DPBS with 2% FBS at 300g for 10mins at 4°C. NK cells were stained with antibodies from Biolegend Inc. San Diego, CA unless otherwise stated. Fluorochrome conjugated antibodies to human CD3 (555275, Becton Dickenson, Franklin Lakes, NJ) CD56 (362514), CD16 (Becton Dickenson, Franklin Lakes, NJ) and CD107a (Becton Dickenson, Franklin Lakes, NJ) were used at a 1/100 dilution. Cells were incubated with antibodies for 20 minutes at room temperature in static conditions in the dark. Flow cytometry collection was performed on BD LSR Fortessa X-20 and analyzed using FlowJo Inc software.

Murine aortic interposition grafting

Murine aortic interposition grafting was performed by Mrs. Winnie Enns as described in Siren et al 2021. Briefly, segments of abdominal aorta from Balb/c donor mice were interposed into the resected infrarenal aorta of C57Bl/6 mice. Syngenic controls consisted of arteries from C57Bl/6 donor mice transplanted into C57Bl/6 recipient mice. CSE modification of donor allograft arteries involved artery perfusion and incubation with 0.5ml of 0.5mM LPG-Sia, 3mM GSH, 5mM $CaCl_2$, and 0.2U/ml gtTGase in UW solution for 30 minutes. Unmodified mouse allograft controls were perfused with GSH, $CaCl_2$, and gtTGase in UW solution without LPG-Sia. Total ischemic time for each transplant was less than 30 minutes. Arteries were collected 15 days post-transplantation for further analysis.

Immunohistochemistry

Cross sections of paraformaldehyde fixed mouse transplanted arteries were rehydrated and underwent heat-mediated antigen retrieval for 30 minutes in a pressure cooker at 121°C. Sections were stained with a primary rabbit polyclonal antibody to CD31 (1:50, AB28364; Abcam, Cambridge, England). After incubations with the primary antibody, sections were incubated with biotin conjugated secondary antibodies, then with horseradish peroxidase-conjugated avidin at room temperature. To develop the substrate, AEC substrate-chromagen (Vector Laboratories, Burlingame, CA) was used to visualize red colored positive staining of the antibodies. Counterstaining with hematoxylin was done to visualize nuclei. Damage to endothelial cells was quantified by determining the amount loss of CD31 staining in the lumen of arteries. Each variable corresponds to one biological replicate from one section of a transplanted artery.

$$100 - \left(\frac{total\ amount\ of\ CD31}{circumference\ of\ the\ artery} \times 100\right) = \%\ of\ area\ without\ CD31\ stain$$

Statistical analysis

A two tailed Student's t-test was performed to determine significant differences between groups. A p-value of ≤0.05 was chosen to be significant.

2.3. Results

LPG-Sia prevents leukocyte-mediated cytotoxicity

To examine how CSE modification of ECs affects immune cell targeting of ECs, a leukocyte cytotoxicity assay was developed. In this assay, IL-2 activated PBMCs were co-cultured with cell surface modified ECs, and EC cell death was measured (Figure 2.1A). Pro-inflammatory IL-2 binds to its receptor T cells and NK cells. IL-2 stimulates T cell proliferation and activation. Memory CD8 T cells stimulated this way can kill target cells in an antigen independent manner. IL-2 binding on NK cells causes NK cells to upregulate expression of activating receptors, such as NKG2D, to induce NK cell-mediated cytotoxicity of target cells. A monolayer of human EC line, EAhy.926, was cultured in a 96-well plate for 7 days. Once cells reached 100% confluency, they were cultured for an extra 4 days to enforce formation of the extracellular matrix. ECs were left untreated or treated with TNF-α to induce expression of ICAM-1 and then co-cultured with IL-2 stimulated PBMCs for 20 hours. To measure immune cell-mediated killing, prior to co-culturing with PBMCs, ECs were labelled with a cell impermeable fluorescent dye, Cell Tracker Green^{TM}. EC death results in loss of membrane integrity and release of fluorescence in the supernatant that can be quantified with a fluorometer. ECs stimulated with TNF-α and co-cultured with IL-2 stimulated PBMCs underwent significantly more immune-mediated EC death as compared to untreated EAhy.926 cells. Also, TNF-α stimulated EAhy.926 cells co-cultured with unstimulated PBMCs resulted in minimal low endothelial cell death (Figure 2.1B; $p < 0.05$).

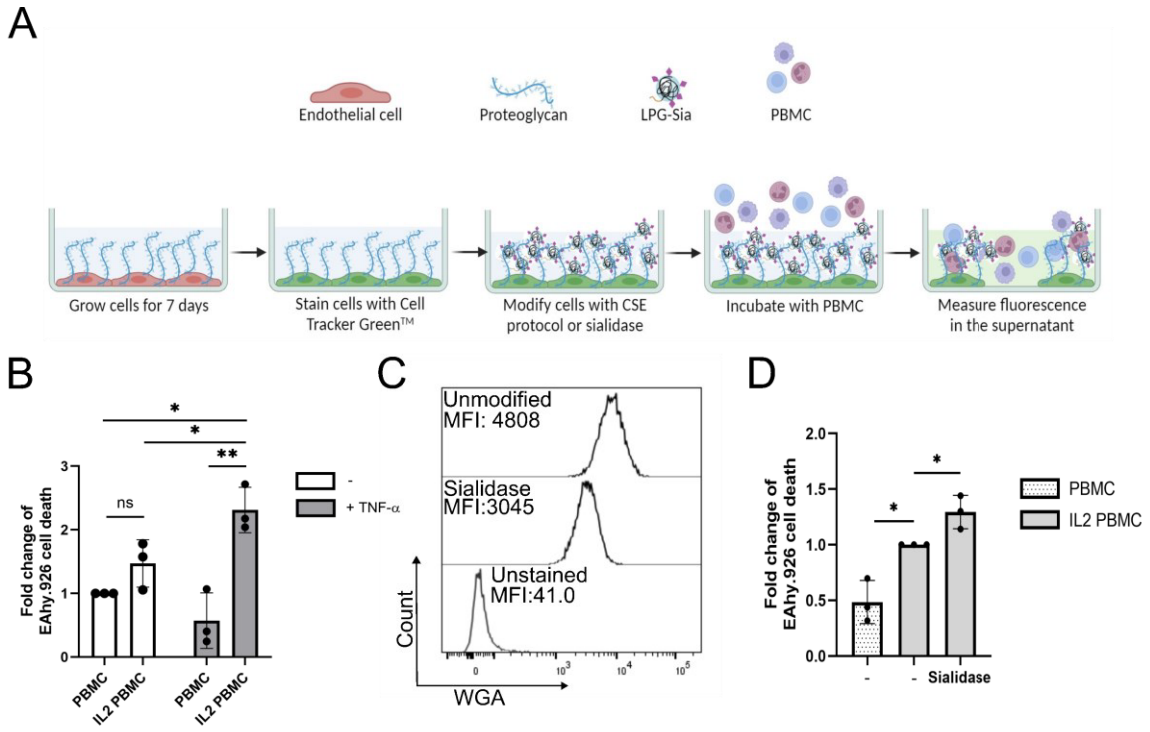


Figure 2.1. Endogenous sialic acid protects endothelial cells.
A. PBMC cytotoxicity assay workflow created with BioRender. **B.** EAhy.926 cells stimulated with TNF-α (10ng/ml) were co-cultured with IL-2 (1000IU/ml) stimulated PBMCs (10:1 effector to target) at 37°C and 5% CO2 for 20hrs. Fluorescence in the supernatant was measured (485/530λ) to determine endothelial cell death. **C.** EAhy.926 were unmodified or modified with Micromonospora viridifaciens sialidase (0.05ug/ml) and stained for sialic acid with FITC fluorochrome conjugated wheat germ agglutinin (2uM) for 1hr at room temperature. **D.** PBMC cytotoxicity assay with unmodified or sialidase modified co-cultured with IL-2 stimulated PBMCs. *, $p < 0.05$ **, $p < 0.005$.

Since sialic acid is abundantly expressed on ECs, I sought to determine whether removal of sialic acid would increase immune-mediated EC death. Sialic acid was removed on the surface of ECs by incubating ECs with 0.05ug/ml sialidase in serum-free media for 1 hour at room temperature. To determine whether the sialidase modification was successful, unmodified or sialidase modified EAhy.926 cells were fluorescently stained with a sialic acid binding lectin, wheat germ agglutinin (WGA), and subjected to flow cytometry. The mean fluorescence intensity (MFI) of sialidase modified ECs was lower compared to unmodified ECs (Figure 2.1C). When ECs were modified with sialidase and co-cultured with IL-2 stimulated PBMCs, EC killing was significantly increased compared to unmodified ECs (Figure 2.1D). This indicated that endogenous sialic acid is protective for ECs from immune cell-mediated killing.

Because removal of endogenous sialic acid increases immune-mediated EC death, I sought to determine whether modifying ECs with LPG-Sia would decrease EC

death. PBMC cytotoxicity assays were performed with PBMC from two male donors on EAhy.926 cells. ECs were modified with sialidase or with LPG-Sia and were co-cultured with IL2 activated PBMCs for 20 hours at 37°C and 5% CO_2. Results showed a significant increase in EC death when ECs were modified with sialidase as compared to the unmodified control (Figure 2.2A; $p<0.05$). LPG-Sia modification resulted in a significantly reduced amount of EC death when compared to both sialidase modified ECs and the unmodified control (Figure 2.2A; $p<0.005$).

To examine whether sialic acid from LPG-Sia is needed for the protective effect of this polymer, ECs were modified with LPG-Sia and then untreated or treated with sialidase. Flow cytometry on modified EAhy.926 cells were performed to quantify sialic acid. Modifying ECs with LPG-Sia led to a slight increase in WGA staining as compared to unmodified control (Figure 2.2C). Treatment of LPG-Sia modified cells with sialidase significantly reduced sialic acid levels (Figure 2.2C). To investigate whether sialic acid on LPG-Sia specifically prevents immune cells from killing ECs, cytotoxicity assays were performed with LPG-Sia modification followed by sialidase. As expected, sialidase modification of ECs resulted in a significant increase of EC death whereas LPG-Sia demonstrated a significant decrease of EC death as compared to the unmodified control (Figure 2.2D; $p<0.05$). Removal of sialic acid from LPG-Sia modified ECs completely prevented the protective capability of the CSE modification (Figure 2.2D; $p>0.05$). These results demonstrated that sialic acid on LPG-Sia is needed to prevent immune cell activation and prevent immune-mediated cytotoxicity of ECs.

To determine if CSE protects ECs from injury in transplant blood vessels, CSE modified transplanted mouse arteries were stained with an endothelial cell marker, CD31, to visualize the integrity of the monolayer (Figure 2.2B). The total length of CD31 expression was calculated in each artery as a percentage of the circumference then subtracted from 100% to determine the percentage of areas lacking CD31 expression, which is reflective of endothelial injury. In syngraft controls, CD31 expression uniformly outlined the entire lumen. UW Control arteries demonstrated increase in endothelial injury, and this was reduced in LPG-Sia modified arteries (Figure 2.2B; $p<0.005$). Together these results demonstrated that LPG-Sia protects ECs from immune-mediated injury and prevents immune cells from targeting ECs in-vitro.

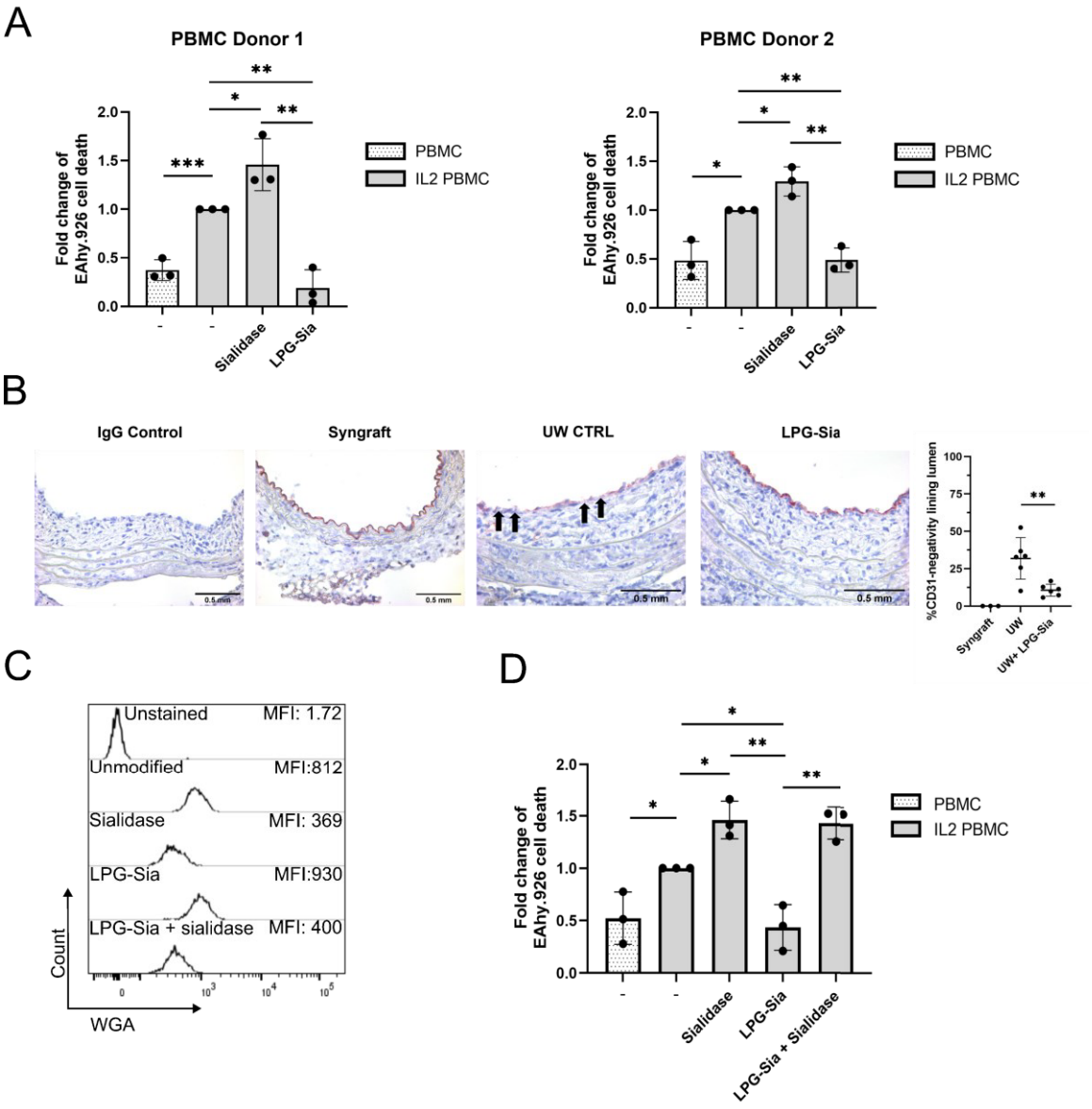


Figure 2.2. CSE modification of endothelial cells with LPG-Sia prevents immune-mediated cytotoxicity.

A. PBMC cytotoxicity assays with EAhy.926 cells modified with viral sialidase or with LPG-Sia (0.5mM LPG-Sia, 3mM GSH, 5mM CaCl2, and 0.2U/ml gtTGase) for 30 minutes 4°C. **B.** CD31 immunohistochemistry of allograft CSE modified arteries 15 days post-transplantation (arrows indicate unstained regions). **C.** Flow cytometry analysis of unmodified or modified EAhy.926 cells stained with FITC-WGA. **D.** PBMC cytotoxicity assay of modified EAhy.926 with LPG-Sia and viral sialidase. *, $p < 0.05$ **, $p<0.005$ ***, $p<0.0001$.

LPG-Sia prevents NK cell mediated cytotoxicity

Because NK cells are the main innate immune cell that causes early injury of transplant blood vessels, I developed a in-vitro model that examines NK cell-mediated cytotoxicity of ECs. To upregulate the expression of NKG2D on NK cells, they were stimulated with IL-2 and IL-15 since the combination of these two inflammatory cytokines was shown to increase the expression of NKG2D and increase cytotoxicity on $CD3^{-}CD56^{+}$ NK cells (Yanping Li et al., 2022; Vuletić et al., 2020). For this model, HUVECs were used because they express higher levels of Mic-A/B on the cell surface than EAhy.926 cells (Riederer et al., 2010). In addition, to increase expression of MIC-A/B on ECs, these cells were stimulated with TNF-α, to upregulate ICAM expression and interferon-β (IFN-β) to upregulate MIC-A/B expression. Activated NK cells were co-cultured with Cell Tracker Green™ labelled ECs and incubated for 20 hours at 37°C and 5% CO_2. ECs were modified with LPG-Sia and/or sialidase using the CSE protocol. Results showed a significant increase in EC death when NK cells were stimulated with IL2 and IL15 as compared to the unstimulated control (Figure 3A; $p<0.05$). This suggested that NK cell mediated cytotoxicity towards ECs was enhanced when NK cells were activated by IL2 and IL15. Removal of endogenous sialic acid with sialidase treatment significantly increased NK mediated killing of ECs (Figure 2.3A; $p<0.05$), which highlights the protective aspect of endogenous sialic acid on ECs. In addition, modifying ECs with LPG-Sia significantly reduced EC death compared to the unmodified control (Figure 2.3A; $p<0.005$). This indicated that LPG-Sia inhibits NK cell effector functions towards ECs.

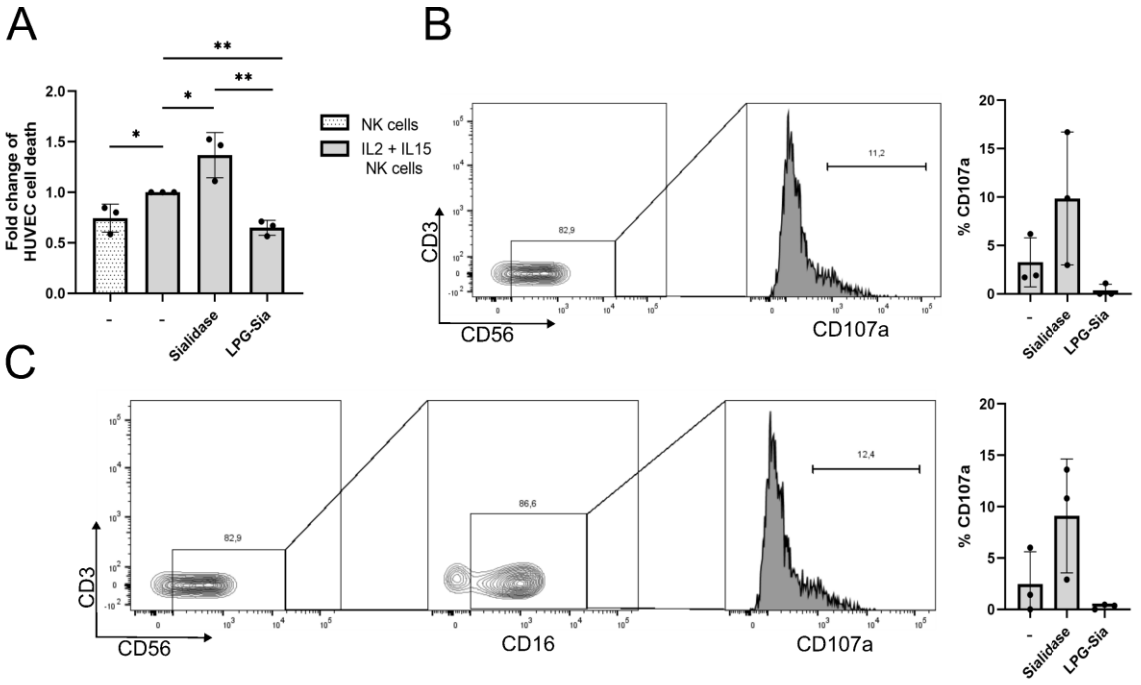


Figure 2.3. LPG-Sia prevents NK cell-mediated cytotoxicity towards ECs.
A. NK cell cytotoxicity assay with HUVECs. NK cells were isolated from PBMCs and stimulated with IL-2 (1000IU/ml) and IL-15 (50ng/ml) then co-cultured with HUVECs stimulated with TNF-α (10ng/ml) and IFN-β (2000IU/ml) for 20hrs at 37°C and 5%CO_2. **B** and **C.** Flowcytometry plots of NK cells from NK cell cytotoxicity assays stained with fluorochrome conjugated antibodies to CD3, CD56, CD16 to measure CD107a externalization. *, $p < 0.05$ **, $p<0.005$.

To investigate whether LPG-Sia can inhibit the degranulation of cytotoxic granules in NK cells, these cells were collected after co-culture with ECs and CD107a externalization was quantified using flow cytometry. When NK cells degranulate cytotoxic granules from intracellular vesicles, they externalize CD107a found in those vesicles onto their cell surface as those vesicles fuse with the plasma membrane. Flow cytometric analysis of $CD3^{-}CD56^{+}$ and $CD3^{-}CD56^{+}CD16^{+}$ cells showed an apparently higher level of CD107a expression when NK cells were co-cultured with sialidase modified ECs (Figures 2.3B and 3C). In addition, when NK cells were co-cultured with CSE modified ECs, CD107a expression was quantitatively lower (Figures 2.3B and 2.3C). However, these findings did not reach statistical significance likely due to variability between the data sets and low sample size.

Protection of ECs from innate immune cell killing by LPG-Sia requires siglec-7 and siglec-9

Sialic acid can bind to receptors on immune cells, termed siglecs, which can cause immune cell activation or deactivation. Inhibitory siglecs contain ITIMs, that initiate inhibitory signal transduction pathways. Both siglec-7 and siglec-9 bind to α-1,3 sialic acid, are expressed on NK cells, and have intracellular ITIMs capable of immune cell deactivation. Since LPG-Sia presents sialic acid with an α-1,3 linkage, both siglec-7 and siglec-9 could bind to LPG-Sia. Therefore, I examined whether siglec-7, siglec-9, or a combination of both induces immune cell deactivation.

Neutralizing antibodies to siglecs-7 and -9 were added to cytotoxicity assays on CSE modified ECs co-cultured with PBMCs. Controls for this assay included unmodified ECs co-cultured with IL-2 activated PBMCs treated with an antibody cocktail of non-specific mouse IgG2a isotype, siglec-7, and siglec-9 to confirm that there was no significant killing induced by non-specific binding of antibodies to Fc receptors. Results showed no significant difference between unmodified ECs combined with IL-2 activated PBMCs and the control antibody cocktail (Figure 2.4). When LPG-Sia modified ECs were co-cultured with IL2 activated PBMCs and siglec-7 neutralizing antibody, there was a numeric but not statistically significant increase in EC death when compared to the LPG-Sia only group (Figure 2.4). However, when siglec-9 neutralizing antibody was added to LPG-Sia modified ECs co-cultured with IL2 PBMCs, there was a significant increase in EC death compared to the LPG-Sia only group (Figure 2.4; $p<0.05$), although this remained less than cell death of unmodified ECs. When both siglec-7 and siglec-9 antibodies were added, there was a significant increase in EC death that was comparable to the unmodified control, indicating that the protective effect of LPG-Sia was completed prevented (Figure 2.4). This indicated that LPG-Sia binds to both siglecs-7 and -9 to deactivate immune cells from targeting ECs for death.

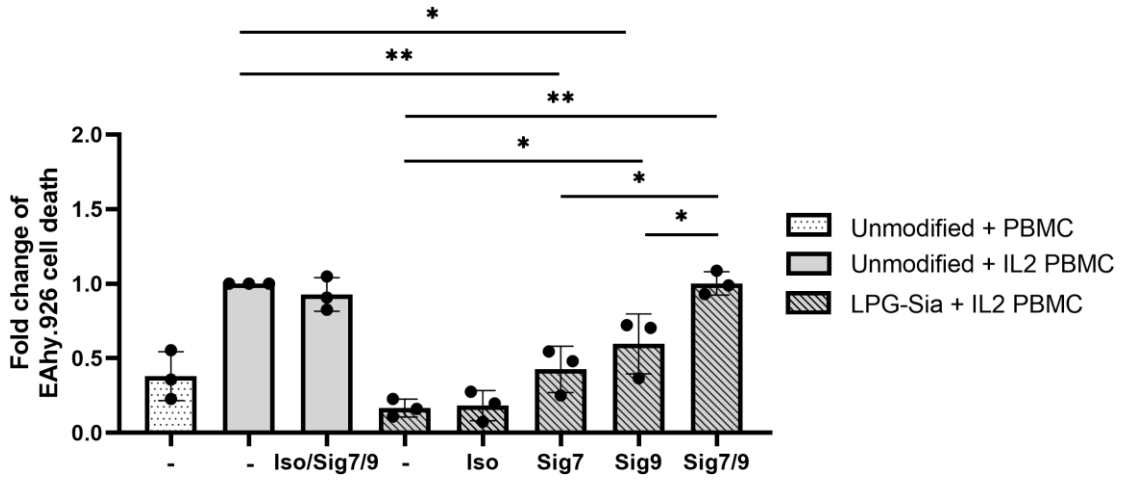


Figure 2.4. LPG-Sia binds to siglec-7 and siglec-9 to deactivate immune cells and prevent immune-mediated endothelial cell death.

PBMC cytotoxicity assay with unmodified or LPG-Sia modified EAhy.926 cells combined with neutralizing siglec-7 or siglec-9 antibodies (10ug/ml) or IgG2a mouse isotype control (10ug/ml) for 20hrs at 37°C and 5%CO_2. Conditions with multiple antibodies were halved or divided into thirds for a total concentration of 10ug/ml. *, $p < 0.05$ **, $p<0.005$.

2.4. Discussion

I have demonstrated that LPG-Sia is immunoregulatory and deactivates PBMCs from causing immune-mediated EC death. Specifically, the sialic acid molecule on LPG-Sia inhibits innate immune cells including NK cells from causing EC death. The validity of the PBMC cytotoxicity assay was confirmed when removal of endogenous Sia demonstrated a significant increase in EC death compared to the unmodified control. It was not surprising to see this increase in EC death as the protective role of Sia on ECs has been well documented. What was surprising was the amount of EC death when ECs were modified with LPG-Sia then sialidase. From Siren et al, there was evidence that LPG itself prevents leukocyte adhesion to ECs (which could contribute to a reduction in EC death), this effect was enhanced upon the addition of Sia. So, I expected that modification of ECs with LPG-Sia then sialidase would result in EC death comparable to the unmodified control rather than the sialidase group. This may have been a result of sialidase removing all available sialic acid, including LPG-Sia and endogenous sialic acid. To provide further evidence that sialic acid on LPG-Sia specifically causes leukocyte deactivation, another experimental group could be added where ECs are modified with LPG only which would result in EC death comparable to the unmodified control.

NK cytotoxicity assays demonstrated that LPG-Sia prevents NK cells from targeting ECs for death. These assays involved stimulation of NK cells with IL-2 and IL-15, which increases the expression of NKG2D. The mechanism by which NK cells cause EC death is likely by NKG2D receptors on NK cells that bind to Mic-A ligands on HUVECs causing release of perforin/granzyme cytotoxic granules (Yanping Li et al., 2022; Vuletić et al., 2020). Although the upregulation of NKG2D by IL-2 and IL-15 stimulation as well as the high expression of MIC-A on HUVECs has been well documented, I could not confirm whether LPG-Sia could prevent CD107a externalization due to high variability within the data. However, more experimental replicates can resolve this issue.

By neutralizing both siglec-7 and siglec-9, the protective effect of LPG-Sia was lost. This indicated that LPG-Sia binds to both siglec-7 and siglec-9 which is in line with their linkage specificity of α1,3-linked sialic acid. Neutralizing siglec-7 did not cause as much loss of the protective effect of LPG-Sia as neutralizing siglec-9. This could be a result of the way sialic acid is presented on LPG-Sia or this could be a specific affect seen only in this donor. To elaborate, donor specific characteristics could include higher expression of siglec-9 than siglec-7 in this donor. Conversely, LPG-Sia could bind to siglec-9 preferentially; however, this is unlikely because siglec-9 binds to sulfated α1,3-linked Sia. An important takeaway from this finding is that since LPG-Sia may bind to both siglec-7 and siglec-9, it has the capability to address donor to donor variability in their expression of these siglecs.

Future experiments are needed to provide evidence of the protective role of CSE modification of EC with LPG-Sia. I only examined leukocytes from male donors because of availability. Future experiments must involve female donors to determine whether there are sex differences. Experiments from Siren et al. provided evidence towards the protection of ECs by LPG-Sia from CD8 T cells, so it is important to develop a CD8 T cell cytotoxicity assay to determine whether LPG-Sia can deactivate T cells. This is essential as CD8 T cells play a major role in causing irreversible damage to allograft arteries. In addition, whether LPG-Sia relies on dual binding of siglec-7/9 is still unconfirmed and experiments with donors with varying siglec-7/9 expression could be performed to conclude that donors must express both receptors for LPG-Sia to confer its immunoregulatory properties. As well, NK cytotoxicity assays must be performed with neutralizing antibodies to siglec-7 and siglec-9 to confirm whether LPG-Sia is deactivating

NK cells by binding to siglec-7 and/or siglec-9 to prevent externalization of CD107a. This would provide a cell-specific mechanism of immune cell regulation by LPG-Sia.

In total, these results provide a possible mechanism for how LPG-Sia downregulates immune cell cytotoxicity towards ECs. LPG-Sia can bind to siglec-7 and siglec-9 to downregulate immune-mediated EC death. Since LPG-Sia binds to both siglecs, LPG-Sia has the potential to address donor to donor variability in transplanted grafts. These results reinforce the establishment of the CSE protocol in the prevention of organ transplant rejection.

Chapter 3. LPG-Sia prevents DC activation and immune cell infiltration in mouse allograft arteries

3.1. Introduction

An important aspect of organ transplant rejection involves the specific targeting of the donor graft by the adaptive immune system which requires activation of T cells by donor and recipient antigen presenting cells. DCs and macrophages express co-stimulatory molecule CD86 that provides co-stimulation needed to activate T cells to migrate to transplanted organs and cause rejection. (Tellides & Pober, 2007).

Shedding of the glycocalyx during the transplantation procedure causes loss of endothelial protection and promotes inflammation (Cancel et al., 2016; Sladden et al., 2019). Because inflammation activates antigen presenting cells and enables them to activate T cells, I hypothesized that CSE modification of transplanted mouse arteries could prevent APCs (DCs and macrophages) from expressing co-stimulatory molecules, such as CD86, and would reduce T cell activation. To study this, a flow cytometry panel was developed to examine myeloid APCs and their expression of CD86 within the grafts. Identification of myeloid APCs involved separating macrophages and matured monocytes, $CD11b^+$ DCs, and conventional DCs in $MHCII^+$ cells. Macrophages and matured monocytes were characterized as $MHCII^+CD11b^+CD11c^-$, $CD11b^+$ "myeloid-derived" DCs were characterized as $MHCII^+CD11b^+CD11c^+$, and conventional DCs were characterized as $MHCII^+CD11b^-CD11c^+$. These classifications were determined from myeloid isolation experiments from Misharin et al. where mouse lungs were digested using an enzyme digestion cocktail (Choi et al., 2011; Misharin et al., 2013). From each subset, the level of CD86 was quantified to identify differences between syngrafts and allografts. Similarly, recipient spleen and lymph nodes were analyzed with the same panel. I found that CD86 expression was increased in conventional DCs in allografts compared to syngrafts. In addition, the amount of CD86 expression in conventional DCs was significantly decreased in CSE modified mouse transplanted arteries. Accumulation of macrophages, CD4 and CD8 T cells, and CD107a expression were reduced when transplanted arteries were modified with the CSE protocol.

3.2. Materials and methods

Murine aortic interposition grafting

Murine aortic interposition grafting was performed by Mrs. Winnie Enns as described in Siren et al 2021. Briefly, segments of abdominal aorta from Balb/c $H2^d$ donor mice were interposed into the resected infrarenal aorta of C57Bl/6 $H2^b$ mice. Syngenic controls consisted of arteries from C57Bl/6 donor mice transplanted into C57Bl/6 recipient mice. CSE modification of donor allograft arteries involved artery perfusion and incubation with 0.5ml of 0.5mM LPG-Sia, 3mM GSH, 5mM $CaCl_2$, and 0.2U/ml gtTGase in UW solution for 30 minutes. Unmodified mouse allograft controls were perfused with GSH, $CaCl_2$, and gtTGase in UW solution without LPG-Sia. Total ischemic time for each transplant was less than 30 minutes. Arteries were collected 5 days post-transplantation for further analysis.

Myeloid cell isolation from transplanted mouse arteries, lymph nodes and spleen

Transplanted allograft and syngraft mouse arteries were collected and subjected to enzymatic digestion at 5 days post-transplantation. As described in Von Rossum et al., 2016 arteries were incubated in 500uL of DPBS with 2mM HEPES supplemented with 60IU/ml DNAseI (10104159001, Sigma-Aldrich, Burlington, MA), 400IU/ml collagenase I (SCR103; Sigma-Aldrich, Burlington, MA), 120IU/ml collagenase XI (C7657; Sigma-Aldrich, Burlington, MA), and 60IU/ml hyaluronidase (H3506; Sigma-Aldrich, Burlington, MA). Arteries were incubated at 37°C and 5% CO2 for 2-3 hours until they became sheared. The suspension was centrifuged at 300g for 10mins at 4°C then resuspended in DPBS with 2% FBS for flow cytometry analysis.

Lymph nodes and spleen from transplant recipients were pulverized using a 0.5ml syringe and 40uM cell strainer into a 6-well plate with 2mls of DPBS. The cell suspensions were transferred to a 15ml conical tube and topped-up to 10mls with PBS and washed at 400g for 5 minutes. Cells were resuspended in 7mls of Red Blood Cell Lysis Buffer (00-4333-57, Thermofisher Science, Waltham, MA) for 7 minutes at room temperature. Cells were then washed at 400g for 5 minutes after adding 8 mls DPBS + 2% FBS. Cells were resuspended in DPBS+ 2% FBS for flow cytometry analysis.

Flow cytometry

Flow cytometry of myeloid cells isolated from mouse artery transplants involved anti-mouse antibodies to CD11b (101257), CD11c (117306), MHC II-IA^{d} (115010), MHC II-IA^{b} (116407), and CD86 (105116) at a 3/100 dilution. Cells were incubated for 20 minutes at room temperature at static conditions without light. Data was acquired on BD LSR Fortessa X-20 and analyzed using FlowJo Inc software.

Immunohistochemistry

Cross sections of paraformaldehyde fixed mouse transplanted arteries were rehydrated and underwent heat-mediated antigen retrieval for 30 minutes in a pressure cooker at 121°C. Sections were stained using rat monoclonal antibodies to CD8 (1:50, 14-0808-82; eBioscience, San Diego, CA), Mac-3 (1:50, 550292; Becton Dickenson, Franklin Lakes, NJ), CD4 (1:50, 14-9766-82; eBioscience), CD107a (1:100, 14-1071-82; eBioscience), and FoxP3 (1:100, 14-5773-80; eBioscience). After incubations with the primary antibody, sections were incubated with biotin conjugated secondary antibodies, then with horseradish peroxidase-conjugated avidin at room temperature. To develop the substrate, AEC substrate-chromagen (Vector Laboratories, Burlingame, CA) was used to visualize red colored positive staining of the antibodies. Counterstaining with hematoxylin was done to visualize nuclei. The accumulation of macrophages, CD8, CD4, and FoxP3 T cells was determined by manually quantifying the number of positive cells in the intima, media, and adventitia. The accumulation of CD107a was determined by measuring the amount of red pigmentation using ImageJ in the intima, media, and adventitia by setting the threshold from syngraft controls. Each variable corresponds to one biological replicate from one section of a transplanted artery.

Statistical analysis

A two tailed Student's t-test was performed to determine significant differences between groups. A p-value of ≤0.05 was chosen to be significant.

3.3. Results

Detection of myeloid cell activation in transplanted mouse arteries

The expression of CD86 on APCs in allograft arteries and lymphoid organs was examined. Mouse artery transplants were performed, and cells from grafts were examined at 5 days post-transplantation because T cell activation is expected to be maximal at this time (Zhuang et al., 2016). Arteries were digested using an enzyme cocktail composed of collagenase I, collagenase XI, DNAse I, and hyaluronidase to break down fibers and connective tissue allowing for collection of single cell suspensions that can be analyzed by flow cytometry (Tellides & Pober, 2007). Isolated cells from the arteries were stained with fluorochrome-conjugated antibodies to differentiate between $H2^d$ Balb/c donor and $H2^b$ C57Bl/6 recipient cells using anti-MHCII $H2^b$ and $H2^d$ antibodies. Myeloid subsets were differentiated by their expression of CD11b and CD11c, and each subset was analyzed for their activation by the amount of CD86 expression. Characterization of myeloid subsets were as follows from $MHCII^+$ cells: matured monocytes and macrophages were $CD11b^+CD11c^-$, myeloid derived dendritic cells were $CD11b^+CD11c^+$, and conventional dendritic cells were $CD11b^-CD11c^+$. Flow cytometry analysis showed that myeloid APCs could be well separated using these markers and that CD86 expression could be examined in each subset (Figure 3.1). A limitation that was quickly identified was the lack of donor, $H2^d$-$MHCII^+$ cells. Therefore, only recipient ($H2^b$-$MHCII^+$) myeloid subsets were further analyzed for CD86 expression (Figure 3.1). The amount of CD86 expression was calculated by subtracting the amount of CD86 in the fluorescence-minus-one CD86 control.

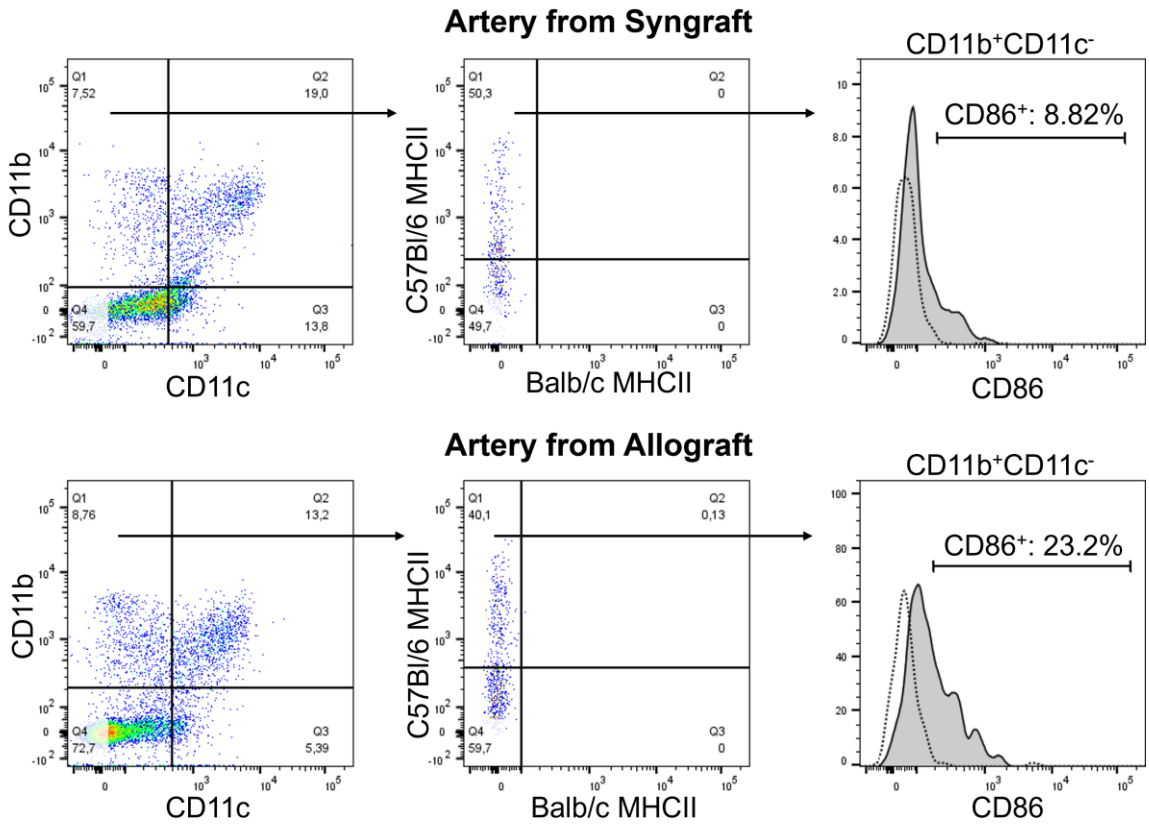
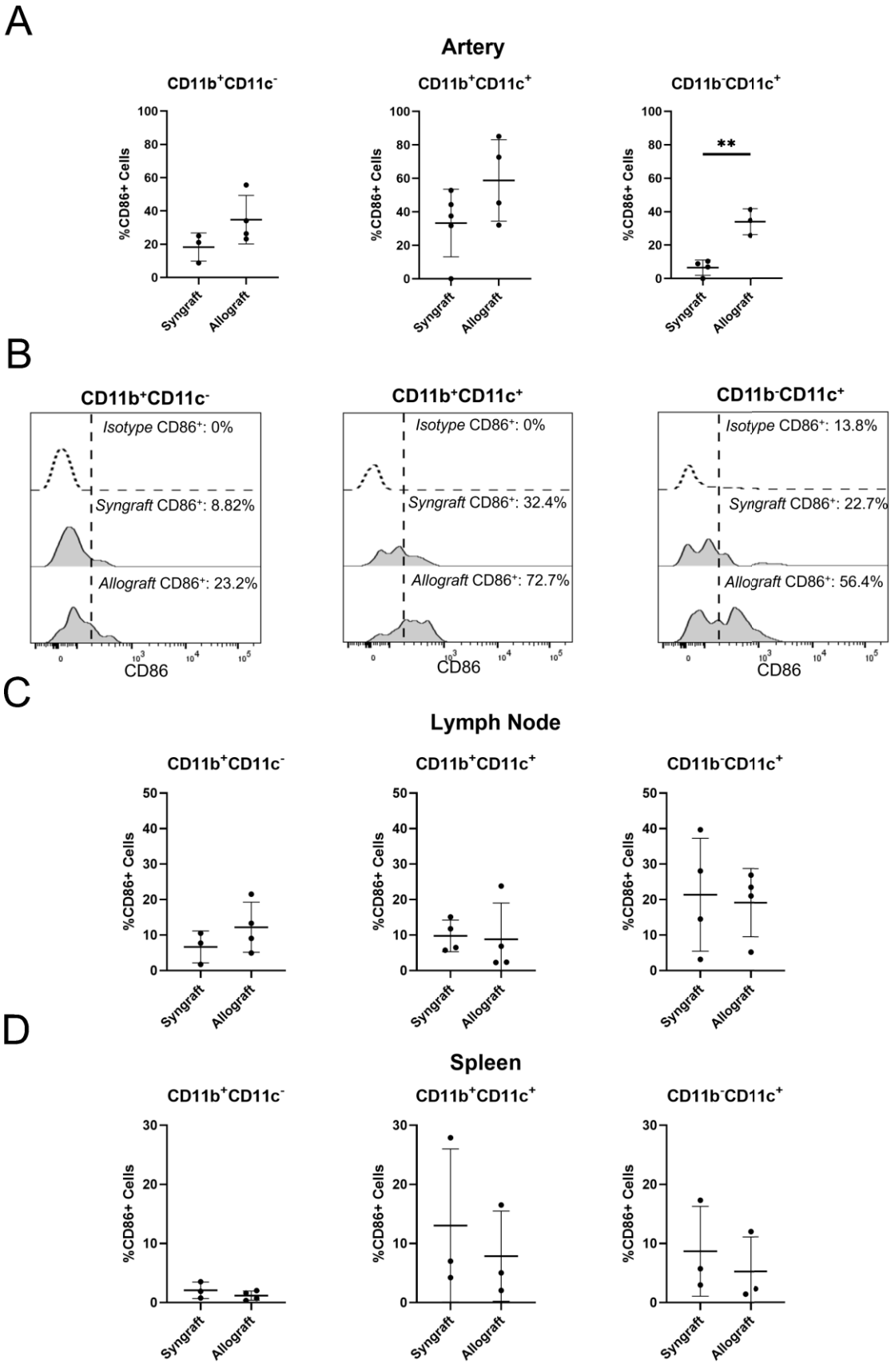


Figure 3.1. Activation of recipient myeloid cells can be detected from syngrafts and allografts 5 days post-transplantation

Transplanted artery segments were enzymatically digested with DNAseI (60U/ml), collagenase I (400U/ml), collagenase XI (120U/ml), and hyaluronidase (60U/ml) in PBS +20mM HEPES for 2-3hrs at 37C + 5% CO2. Cells were stained with fluorochrome conjugated antibodies to donor $H2^d$-MHCII, recipient $H2^b$-MHCII, CD11b, CD11c, and CD86 for 20mins at room temperature and analyzed with flow cytometry. Dashed lines represent fluorescence-minus-one (FMO) CD86 isotype control.

There was a numeric increase in CD86 expression on $CD11b^+CD11c^-$ and $CD11b^+CD11c^+$ myeloid cells from allografts when compared to syngrafts that did not reach statistical significance (Figure 3.2A). In conventional DCs ($CD11b^-CD11c^+$), CD86 expression was markedly and significantly increased in allografts (Figure 3.2A; $p < 0.005$). These results indicate that conventional DCs from recipients infiltrate the graft and are activated to express co-stimulatory molecules that could enable them to activate T cells. To determine whether differences in CD86 expression in these myeloid subsets could also be identified in recipient lymph nodes and spleen, cells from these tissues were subjected to flow analysis with the same flow panel. Variability was high between transplants and resulted showed no differences in CD86 expression in any of the myeloid populations in lymph nodes and spleen (Figure 3.2B and C).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Allograft CD86+: 23.2%
Isotype CD86+: 0%
Syngraft CD86+: 32.4%
Allograft CD86+: 72.7%
Isotype CD86+: 13.8%
Syngraft CD86+: 22.7%
Allograft CD86+: 56.4%
CD86
C
Lymph Node
D
Spleen

Figure 3.2. CD86 expression of myeloid subsets in artery, lymph nodes, and spleen from mouse syngrafts and allografts

CD86 expression of myeloid subsets was determined by measuring total %$CD86^+$ and subtracting %$CD86^+$ in FMO CD86 isotype control in transplanted mouse artery, recipient lymph nodes and spleen. **A**. Arteries were digested with an enzyme cocktail. **B**. Flow cytometry representation of CD86 expression in arteries. **C**. Recipient lymph nodes and **D**. spleen from mouse artery transplants after 5 days. **, $p<0.005$.

The effect of CSE on CD86 expression in cells from artery grafts was then examined. I hypothesized that since sialic acid on LPG-Sia is immunoregulatory, CD86 expression would be decreased in myeloid APCs in CSE modified transplants compared to the UW only controls. Results showed that CSE did not affect CD86 expression in $CD11b^+CD11c^-$ or $CD11b^+CD11c^+$ populations (Figure 3.3). When conventional DCs ($CD11b^-CD11c^+$ cells) were examined, CSE modification substantially and significantly reduced CD86 expression as compared to controls (Figure 3.3; $p < 0.05$). This indicated that LPG-Sia could deactivate or prevent activation of conventional dendritic cells in mouse allografts, which reduces their ability to provide co-stimulation to T cells. Flow cytometric analysis of CD86 expression on myeloid subsets in recipient lymph nodes and spleen was also performed in CSE modified allografts but there were no differences in any myeloid population in these tissues (Supplementary Figure S.2).

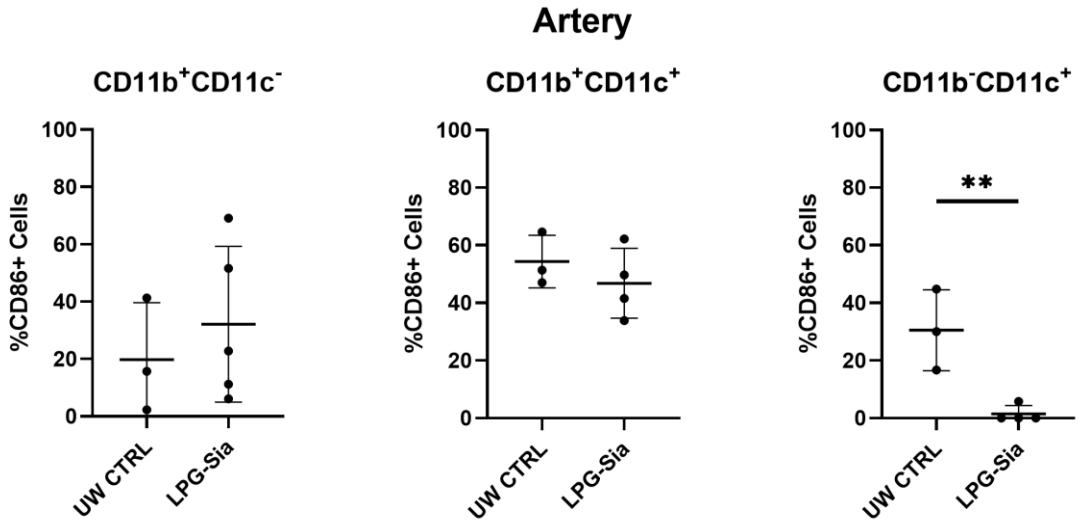
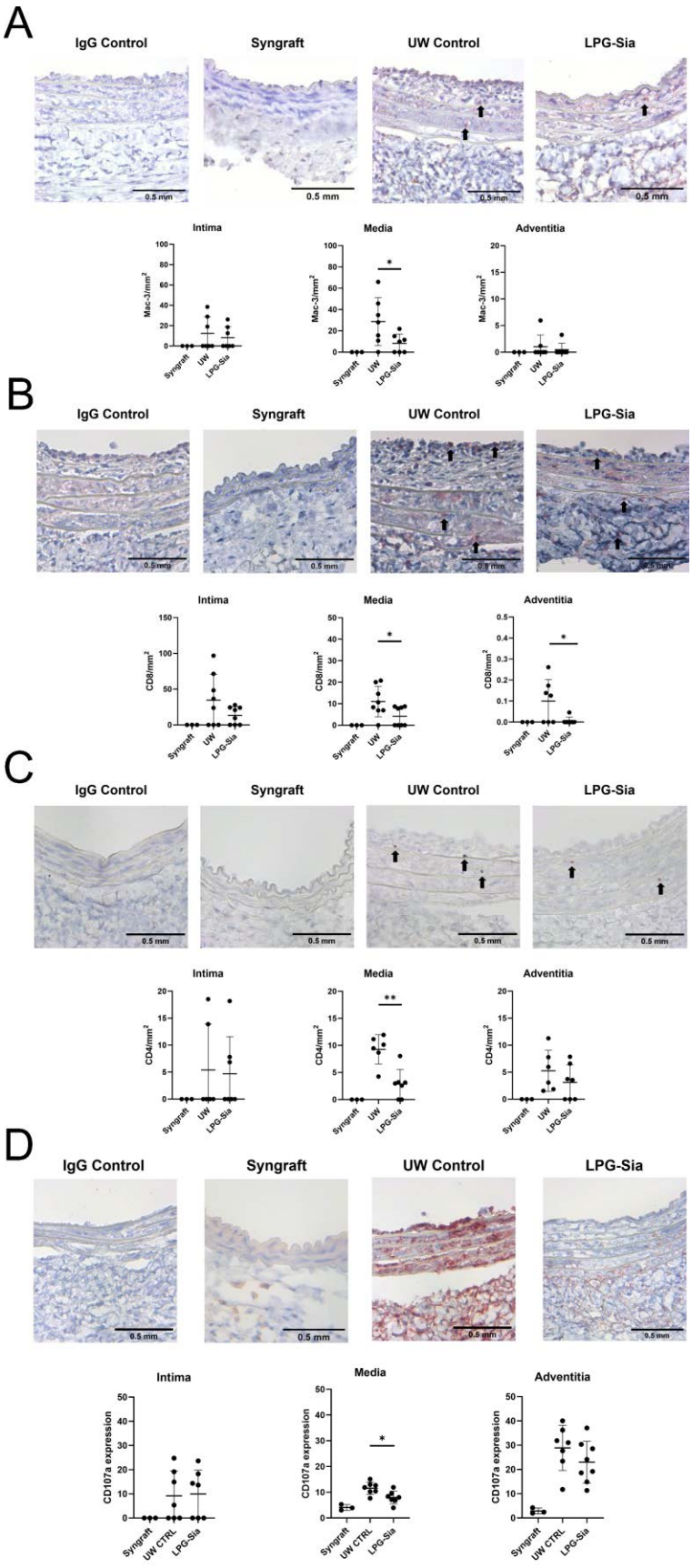


Figure 3.3. CD86 expression is reduced on conventional dendritic cells in CSE modified transplanted mouse arteries.

Transplanted artery segments were enzymatically digested and subjected to flow cytometric analysis. Final %$CD86^+$ was determined by subtracting background %$CD86^+$ in FMO CD86 isotype control from each sample. **, $p<0.005$.

LPG-Sia reduces macrophage, CD8 and CD4 T cell, and CD107a accumulation in mouse artery transplants

Macrophages were detected by immunohistochemical staining for the mouse macrophage and monocyte marker, Mac-3. There was a significant reduction of Mac-3 in the media of CSE modified allograft arteries as compared to the UW only control (Figure 3.3A; $p<0.05$). When CD8 and CD4 T cells were examined, the accumulation of both CD8 and CD4 T cells was reduced in the media and adventitia of CSE modified transplanted mouse arteries (Figure 3.3B and C; $p<0.05$). Cytokine analysis from Siren et al., 2021 showed a significant increase in serum IL-10, which could indicate the presence of Tregs. However, FoxP3+ T regs were exceedingly rare in both experimental groups (Supplemental Figure S.1). Finally, CD107a expression was significantly reduced only in the media of CSE modified allografts as compared to UW Control arteries as expected (Figure 3.3D; $p<0.05$). Because macrophage, CD8 and CD4 T cell infiltration in the media were significantly reduced in CSE modified allografts, this could indicate that there was an overall reduction of T cell-mediated rejection.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Adventitia
Mac-3/mm²
Syngraft
UW
LPG-Sia
*
B
IgG Control
Syngraft
UW Control
LPG-Sia
0.5 mm
Intima
Media
Adventitia
CD8/mm²
Syngraft
UW
LPG-Sia
*
C
IgG Control
Syngraft
UW Control
LPG-Sia
0.5 mm
Intima
Media
Adventitia
CD4/mm²
Syngraft
UW
LPG-Sia
**
D
IgG Control
Syngraft
UW Control
LPG-Sia
0.5 mm
Intima
Media
Adventitia
CD107a expression
Syngraft
UW CTRL
LPG-Sia
*

Figure 3.4 Mac-3, CD4, CD8, and CD107a are reduced in CSE modified mouse allografts

Immunohistochemistry of transplanted mouse allografts were performed 15 days post-transplantation. **A**. Anti-mac-3 antibody to detect macrophages. **B**. anti-CD8 to detect CD8 T cells. **C**. Anti-CD4 to detect CD4 T cells. **D**. Anti-CD107a on non-permeabilized arteries. Black arrows indicate positively stained cells. *, $p < 0.05$, ** $p < 0.005$.

3.4. Discussion

Induction of co-stimulatory molecule expression on myeloid APCs is needed for T cell activation. Understanding whether CSE modification of transplanted mouse arteries can reduce macrophage or DC activity is essential to establish the CSE protocol in the prevention of allograft rejection. My findings indicate that LPG-Sia modification of mouse arteries significantly reduces co-stimulatory activity of recipient conventional DCs. As previous results have shown, sialic acid on LPG-Sia can bind to siglec-9, which is the paralog of siglec-E in mice and is expressed on conventional dendritic cells (Lock et al., 2004). Further experiments should be performed to determine whether CSE may modify other immune molecules on conventional DCs such as programmed death ligand-1 (PDL-1), that would further reduce T cell activation.

It is unclear whether specific APC subsets play distinct roles in inducing T cell activation in transplant rejection. My results indicate that conventional DCs from recipients upregulated CD86 in mouse allografts at 5 days post-transplantation more than macrophages and/or matured monocytes and myeloid-derived DCs. As described in Mishrain et al. conventional dendritic cells can be further separated into $CD103^{+}CD64^{-}CD24^{+}$ DCs to identify $CD103^{+}$ DCs. These DCs have shown to be tolerogenic by expressing PDL-1^{hi} to induce Treg differentiation but can also induce T cell activation through the expression of co-stimulatory molecules CD80/CD86 (Cao et al., 2016; Shiokawa et al., 2017). Therefore, as interest in the development of in-vitro tolerogenic DCs for the prevention of allograft rejection increases, these results point towards a specific dendritic cell subset that can be targeted for therapeutic purposes.

Because CSE modification of mouse allografts prevents DC co-stimulation at 5 days post-transplantation, understanding whether accumulation of macrophages, CD8, CD4, and FoxP3 T cells could be impacted was essential. Results showed a significant decrease in macrophages, CD8 and CD4 T cells in CSE modified mouse allograft which

is reflective of reduced T cell-mediated rejection. Since macrophages and CD8 T cells can exhibit cytotoxicity, reduction of these cells in the graft provides an explanation to the reduction of acute injury in CSE modified allografts. As well, reduction of CD4 T cells in the graft could prevent B cell activation that produces donor specific antibodies. This could provide an explanation for the reduction of donor specific antibodies in response to CSE modified transplants. Further experiments should be performed to determine whether B cell activation is reduced in lymphoid organs. Since results show that sialic acid on LPG-Sia is immune regulatory and conventional DC co-stimulation was reduced 5-days post-transplantation, increased infiltration of $FoxP3^{+}$ Tregs was expected. However, Tregs were rare at 15-days post-transplantation which prevented my ability to assess this. From Siren et al., 2021 increased concentrations of LPG-Sia had stronger immune downregulating capabilities, and all experiments were performed at the lowest concentration which showed a protective affect. Therefore, future CSE modifications could be performed with a higher concentration of LPG-Sia to induce a stronger signal for Treg differentiation by conventional DCs.

These results provide further evidence towards the immune regulatory role of LPG-Sia in the prevention of transplant rejection. LPG-Sia modification of allografts showed reduction of conventional DC co-stimulatory activity and the protective effects of CSE modification of allografts reduces macrophage, CD8 and CD4 T cell infiltration in the graft to reduce vascular injury.

Chapter 4. General discussion

The use of broad acting immunosuppressives in the prevention of organ transplant rejection allows for increased longevity of the graft. However, the adverse risks and side effects associated with immunosuppressives pose a significant threat to the health of transplant recipients. Therefore, the need to develop therapeutic strategies to circumvent the use of immunosuppressives is essential. Current strategies that are being explored include the use of ex-vivo cell therapies to induce tolerance, immunosuppression via nanoparticles to direct immunosuppressants only in the graft, and organ engineering to convert foreign organs to match the recipient using stem cells (Chow et al., 2019; Fisher et al., 2015; Juvet et al., 2014; Trzonkowski et al., 2009). The CSE protocol I have examined reduces both acute and chronic rejection in mouse artery and kidney allografts by enzymatically adhering a sialic acid presenting polymer, LPG-Sia, to ECs on blood vessels. During the organ transplantation process, blood vessels become damaged and exhibit the loss of the protective glycocalyx matrix found on ECs. This matrix can transduce sheer stress and provides a scaffold for immune cell signaling while forming a barrier for ECs to the lumen. The loss of the protective functions of the glycocalyx and its functions is indicative of vascular health. Therefore, understanding the mechanism by which LPG-Sia prevents acute and chronic rejection will establish the CSE protocol in the prevention of organ transplant rejection while circumventing the use of immunosuppressives.

The potential role of glycan associated-sialic acids in allograft rejection

Major components of the glycocalyx include hyaluronan, peptidoglycans, and glycoproteins capped with sialic acid. Sladden et al. 2019 described how lung transplant recipients experiencing primary graft dysfunction exhibited an increase in hyaluronan and donor syndecan-1, a proteoglycan, in peripheral blood of transplant recipients. This paper and others indicated that shedding of glycocalyx components is a biomarker for allograft rejection (Inkinen et al., 2019; Schiefer et al., 2015; Sladden et al., 2019). However, these authors do not measure changes in sialic acid in transplant recipients although sialic acid is an emerging biomarker for cardiovascular disease and heart failure (Knuiman et al., 2004; C. Li et al., 2021). In addition, atherosclerosis is chronic vascular disease initiated by uptake of low-density lipoproteins inhibiting by resident macrophages, eventually resulting in plaque formation and clotting. A recent study describes how desialylation of apolipoprotein B (ApoB) by NEU1 causes an increase of uptake of ApoB by macrophages

to enhance plaque formation (Demina et al., 2021). This indicates a possible role of sialidases and sialic acid in regulating glycocalyx components and preventing plaque formation. From my experiments, IHC of CD31 demonstrated that CSE modification of mouse allografts prevented EC injury. Also, my in-vitro PBMC cytotoxicity assays with sialidase treatment of ECs demonstrated that loss of endogenous sialic acid causes increase in EC death. Together, these results highlight the potential role of sialic acid in preventing EC injury which could be implicated in glycocalyx shedding. Future experiments could involve measuring the amount of sialic acid or sialidase in serum from mouse artery transplants to determine whether there are changes in sialic acid in allografts compared to syngrafts. As well, sialidase treatment of mouse arteries prior to transplantation would provide better insight on immune cells that are impacted by changes in sialic acid content on the glycocalyx in organ transplantation.

The composition of the glycocalyx has been well studied. However, understanding whether the loss of sialic acid could cause changes in glycocalyx composition within the context of other essential components is unclear. Since sialic acid is a negatively charged residue, loss of sialic acid could result in weakening of the electrostatic forces resulting in a cascading effect resulting in low binding of non-transmembrane components. For example, hyaluronan is embedded on the surface of ECs by intercalating with negatively charged membrane proteins such as proteoglycans and binding with high affinity to hyaluronan binding protein-1 (HABP1) which is a sialyated glycoprotein (Fan et al., 2019; Melrose, 2023). Loss of sialic acid could reduce the binding affinity of hyaluronan to negatively charged proteoglycans as well as HABP1 resulting in shedding of hyaluronan. By understanding this, the role of sialic acid in maintaining glycocalyx homeostasis in the context of other components would provide insight into how glycocalyx shedding occurs in allograft rejection. In addition, determining how LPG-Sia impacts the glycocalyx matrix in the context of other components would be essential in understanding how CSE modification of mouse allograft prevents transplant rejection. Future experiments to investigate this would include modifying ECs and measuring changes in the amount of sialic acid, hyaluronan, collagen, and elastin on the glycocalyx and in the media. Understanding this would also provide insight into how CSE modification of mouse allograft arteries reduces accumulation of macrophage, CD4 and CD8 T cell accumulation.

The potential role of siglecs in transplantation

Currently, there is only one review article that discusses the potential role of siglecs in organ transplantation (S. Cai et al., 2019). The results from my experiments provide evidence towards their potential contribution in allograft rejection. In-vitro cytotoxicity assays indicate that sialic acid on LPG-Sia has immunoregulatory properties. More specifically, sialic acid on LPG-Sia downregulates PBMCs and NK cells from targeting ECs for immune-mediated death in-vitro. LPG-Sia can bind to both siglec-7 and siglec-9 on PBMCs to inhibit immune cell cytotoxicity towards ECs. To provide evidence towards endogenous sialic acid acting on siglecs future experiments could involve neutralizing siglecs on PBMCs co-cultured with unmodified ECs or pull-down experiments with ECs and siglec-Fcs. Also, to establish that siglec-7 and siglec-9 bind to LPG-Sia to inhibit immune cell cytotoxicity future experiments involving neutralization of siglec-E, the murine paralog to siglecs-7/9, on CSE modified allografts must be performed.

Does CSE modification of mouse allografts prevent T cell activation?

A recent study investigated whether blocking sialic acid expression on DCs could impact CD8 T cell activation. Results showed an increase in antigen-specific CD8 T cell proliferation and response (Balneger et al., 2022). My ex-vivo experiments show that CSE modification of mouse allografts prevents expression of recipient conventional DC co-stimulatory expression which could prevent T cell activation. J. Wang et al., 2022 describe how sialic acid modulate DC activation and antigen presentation to T cells by binding to siglec-7 and siglec-9. Therefore, it is possible for LPG-Sia to bind to DCs to prevent or inhibit expression of co-stimulatory molecules which could prevent T cell activation. Whether DCs become activated before or after entering the graft is unclear, so how LPG-Sia interacts with recipient conventional DCs remain unknown. In addition, whether T cells in CSE modified mouse allografts have a decreased amount of CD28 expression is unknown. To investigate this similar ex-vivo experiments examining CD28 expression in CSE modified mouse allografts would be performed. Also, in-vitro experiments from Siren et al. 2019 demonstrated that CSE modification of ECs inhibit CD8 T cell-mediated EC death. This indicates that LPG-Sia could bind directly to CD8 T cells. This is in line with a recent discovery which described how sialyated glycans could be ligand for CD28 on T cells and compete with CD80 to deactivate T cells (Edgar et al., 2021). Whether CSE modification of mouse allografts causes T cell deactivation directly by LPG-Sia or by inhibiting expression of co-stimulatory molecules on DCs needed further investigated.

Limitations

Although in-vitro experiments were performed with male blood donors, the in-vivo affects of LPG-Sia modification of mouse allografts does not show sex dependency so it is unlikely that the proposed mechanism is male-dependent. Regardless, future experiments must include female leukocytes to fully establish the role of the CSE protocol in the prevention of allograft rejection. Also, IHC experiments showed reduced accumulation of CD107a in CSE modified mouse allograft arteries, and since CD107a is expressed in all cells on lysosomes it is unclear whether differences in the amount of CD107a indicative of reduced immune cell infiltration. To confirm this finding, future experiments must include IHC of with immune cell marker CD45. Reduction of CD45 in CSE modified and unmodified mouse allograft arteries would provide stronger evidence for this claim. The mechanism proposed in this thesis involve siglec-7 and siglec-9 binding to LPG-Sia resulting inhibition of immune-mediated cytotoxicity of ECs. As discussed earlier, Edgar et. Al. 2021 describes how sialyated glycans can compete with CD80 to prevent T cell activation. Therefore, it is possible that sialic acid on LPG-Sia can bind to other receptors to contribute to immune cell deactivation.

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Appendix. Supplementary Figures

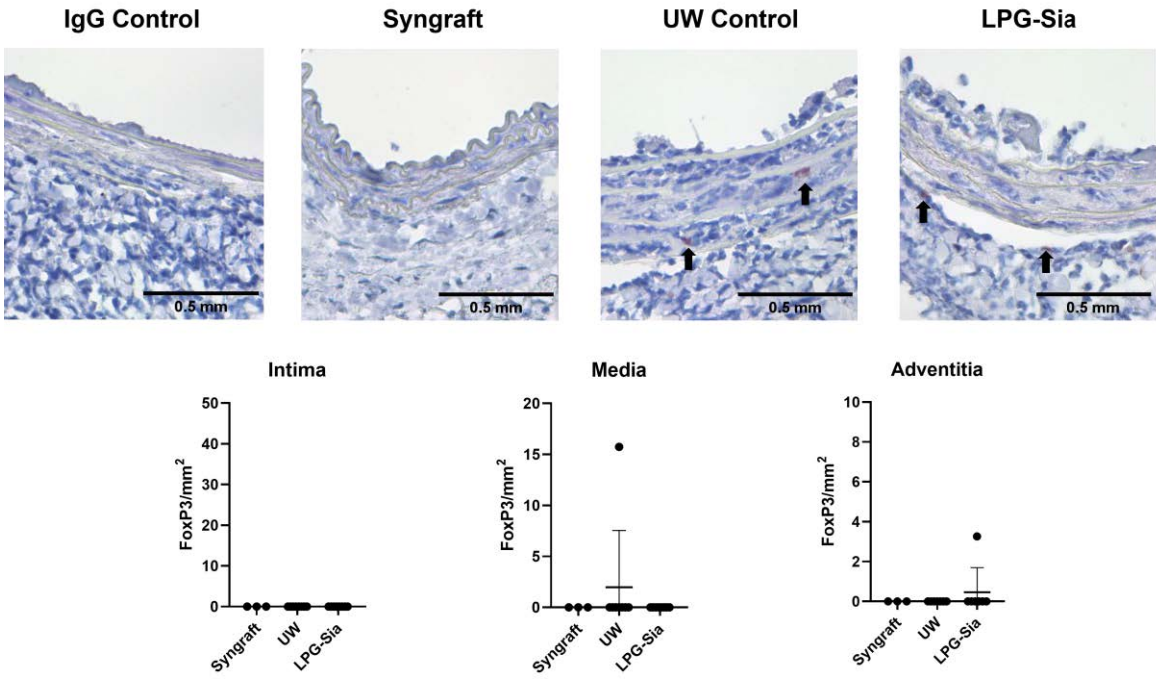

Figure A.1. Low levels of FoxP3 was observed in mouse allografts 15 days post-transplantation.

Immunohistochemistry of transplanted mouse allografts were performed 15 days post-transplantation with anti-FoxP3. Black arrows indicate positively stained cells.

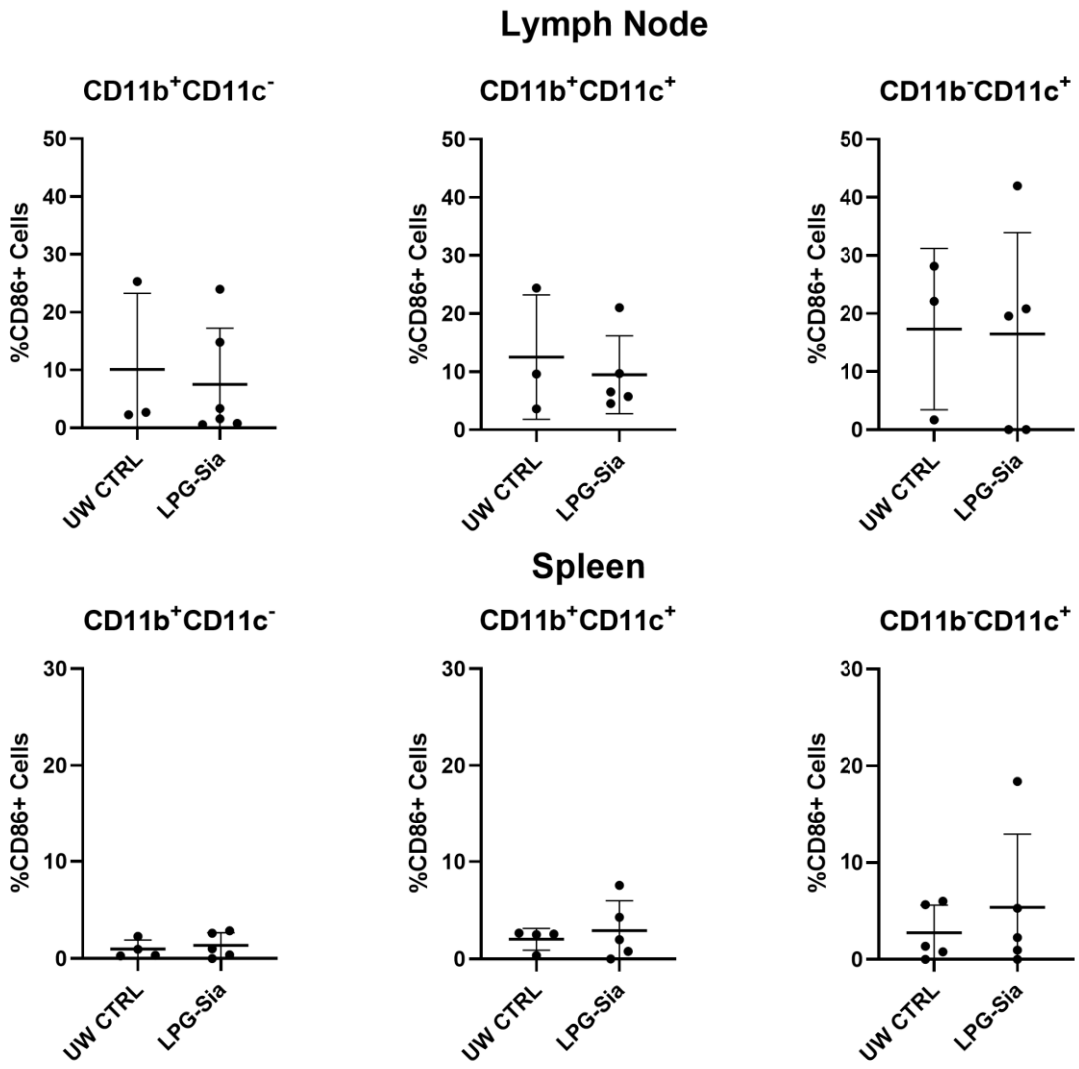

Figure A.2. CD86 expression did not differ in myeloid subsets in lymph nodes and spleen of CSE modified transplants .

CD86 expression was determined by subtracting background CD86 in fluorescence-minus-one CD86 control.