Selective Deletion of Heparan Sulfotransferase Enzyme, Ndst1, in Donor Endothelial and Myeloid Precursor Cells Significantly Decreases Acute Allograft Rejection

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Early damage to transplanted organs initiates excess inflammation that can cause ongoing injury, a leading cause for late graft loss. The endothelial glycocalyx modulates immune reactions and chemokine-mediated haptotaxis, potentially driving graft loss. In prior work, conditional deficiency of the glycocalyx-modifying enzyme N-deacetylase-N-sulfotransferase-1 (Ndst1<sup>f/f TekCre</sup>) reduced aortic allograft inflammation. Here we investigated modification of heparan sulfate (HS) and chemokine interactions in whole-organ renal allografts. Conditional donor allograft Ndst1 deficiency (Ndst1<sup>−/−</sup>; C57BL/6 background) was compared to systemic treatment with M-T7, a broad-spectrum chemokine-glycosaminoglycan (GAG) inhibitor. Early rejection was significantly reduced in Ndst1<sup>−/−</sup> kidneys engrafted into wildtype BALB/c mice (Ndst1<sup>−/−</sup>) and comparable to M-T7 treatment in C57BL/6 allografts (P < 0.0081). M-T7 lost activity in Ndst1<sup>−/−</sup> allografts, while M-T7 point mutants with modified GAG-chemokine binding displayed a range of anti-rejection activity. CD3+ T cells (P < 0.0001), HS (P < 0.005) and CXC chemokine staining (P < 0.012), gene expression in NFκB and JAK/STAT pathways, and HS and CS disaccharide content were significantly altered with reduced rejection. Transplant of donor allografts with conditional Ndst1 deficiency exhibit significantly reduced acute rejection, comparable to systemic chemokine-GAG inhibition. Modified disaccharides in engrafted organs correlate with reduced rejection. Altered disaccharides in engrafted organs provide markers for rejection with potential to guide new therapeutic approaches in allograft rejection.

Acute and chronic transplant rejection, with scarring and organ failure, prolong illness, increase mortality and increase the necessity for repeat transplant<sup>1–7</sup>. Organs available for transplant are limited and there is a large, unmet need for new treatments that reduce transplant vasculopathy and rejection. Early rejection is the leading cause of graft loss in the first year post-transplant, while chronic rejection with allograft vascular disease is a leading cause of late graft loss after the first year post-transplantation and some of this ongoing chronic damage is believed to be initiated early after graft implant. Transplant allograft vasculopathy (TAV) causes graft scarring and late loss associated with chronic rejection. Development of chronic rejection with TAV is induced, in part, by...
both recurrent episodes of acute antibody-mediated immune rejection and also persistent excess inflammation.14–16. Thus, both changes related to cellular rejection and antibody-mediated rejection have the potential to induce early damage to the graft with long-lasting effects. Some of these inflammatory, non-antibody mediated immune responses are produced by surgical and ischemic injury and infection at the time of transplant, occurring early after engraftment with long lasting effects on organ function.17–19

Most treatments for preventing rejection target the immune response of the recipient host and few have investigated directly treating the donor organ prior to transplantation as a method to reduce early damage and ongoing excess inflammation. Local inflammation may be driven by changes in the endothelial layer glycocalyx after injury. Thus, we have postulated that treatments designed to modify the donor tissue glycocalyx content may beneficially alter early innate and acquired immune responses.

Glycosaminoglycans (GAGs) are complex, linear, negatively-charged polymers consisting of repeating subunits of polysaccharide sugars. Heparan sulfate (HS) is the predominant GAG present on the surface of cells, representing a major component of the extracellular matrix, with multiple roles in physiological and pathophysiological processes. GAGs are critical in vascular physiology where they form the glycocalyx, a meshwork of carbohydrates that coats vascular endothelial cells and regulates vascular permeability, acts as a transducer of fluid shear forces, modulates receptor activity and cellular adhesion and/or activation, and provides the substrate for directional chemokine gradient formation to mediate leukocyte chemotaxis and invasion.11–14. GAGs and the endothelial glycocalyx may have a role in tissue graft survival. For example, treatment with a mutant of the CXCL8 (IL-8) chemokine, which has enhanced GAG and reduced chemokine receptor binding, has been reported to reduce early rejection in a rodent transplant model, further supporting a central role for chemokine-GAG interactions in rejection.20–22. In prior work, reductions in HS binding and HS glycoproteins, such as perlecian, modified acute monocyte chemoattractant protein-1 (MCP-1)-mediated monocyte infiltration in renal ischemia reperfusion injury. Blockade of chemokine-GAG interactions using MC2, a peptide derived from the HS-GAG binding domain of IFN gamma (IFN-γ) also reduces inflammation and prolongs dermal graft survival in a mouse model.23–25. The endothelial glycocalyx therefore has fundamental roles in cellular responses in early rejection, whether cellular or antibody-mediated.

Chemokines have proven dual interactions with GAGs and with 7 transmembrane G protein coupled chemokine receptors on immune cells.7,15–19. This requisite GAG and receptor interaction presents one mechanism through which modified GAG composition may alter acute transplant injury and rejection. Chemokines activate cells via surface receptors, however, certain chemokines also unexpectedly signal cell activation through cooperative receptor activation via direct GAG interaction, bypassing receptors.7

While chemokines have been studied extensively in transplants, the role of GAG interactions is less well defined.16–29. As noted, the main tissue GAG is heparan sulfate (HS), but the endothelial glycocalyx also contains other GAGs such as chondroitin sulfate (CS) and hyaluronic acid (HA). GAGs are produced by enzyme-mediated polymerization and can be present as free polysaccharides or associated with glycoproteins. Thus, unlike proteins, changes in GAG composition are less directly linked to gene expression, but instead reflect altered activity of synthetic, metabolizing, and modifying enzyme activity at the tissue level. Given the importance of glycocalyx GAG composition in driving immunological activities in the vasculature and the role these activities play in transplant graft survival, we hypothesize that changes in HS and disaccharides in transplanted grafts may help to identify early or persistent rejection, or even guide new therapeutic approaches to preventing graft rejection.

N-deacetylase-N-sulfotransferase-1 (Ndst1) acts as a central modifying enzyme in HS, catalyzing sulfate conjugation to carbohydrates. Prior work by other researchers has demonstrated that conditional Ndst1 deficiency in endothelial and myeloid precursor cells reduced inflammatory cell invasion, acute antibody-induced nephritis, and allergic airways disease in experimental models.21,22. Our own work with aortic allografts, which is a model for chronic transplant vascular inflammation and fibrosis,20,21, we demonstrated reduced vasculopathy and inflammation at 4 weeks follow-up in Ndst1−/− donor aortas implanted in WT BALB/c mice with normal Ndst1 expression (Ndst1+/+)23. Ndst1 deficiency in acute, early rejection in solid organ transplants has not been previously examined, nor have modifications of GAG composition been assessed. Thus the independent functions of glycosaminoglycans (GAG) in donor organs after transplant, and specifically in the endothelial glycocalyx, are incompletely understood.11–14,16–18,22.

Larger DNA viruses have evolved pan-specific chemokine modulating proteins (CMPs) that inhibit a broad range of chemokines, differing from ligand-specific chemokine antagonists.15,19,23–30. M-T7 is a 37 kDa Myxomavirus-derived secreted glycoprotein that possesses both broad spectrum, species-independent, C, CC and CXC chemokine inhibitory activity and a rabbit species-specific interferon gamma (IFN-γ) inhibitory activity.31–34. M-T7 point mutations have also been developed with variably modified GAG and chemokine interactions.35,36. M-T7 binds the GAG-binding domain of chemokines from multiple species, thereby disrupting diverse classes of chemokine gradients. M-T7 activity was blunted in Ndst1-deficient mouse donor aortic transplants, a model for chronic TAV and vascular injury, but not in CC chemokine receptor deficient aortic allograft transplants, supporting M-T7 interference with chemokine-GAG interactions.37. Treatment with purified M-T7 protein reduces mononuclear cell invasion and intimal plaque in rodent models of angioplasty injury, as well as in aortic and renal transplants, with improved long term (>100 days) renal allograft survival and reduced scarring. M-T7 treatment effects on acute rejection have not as yet been examined.19,23,26–29.

We investigate here the effect of isolated donor organ Ndst1 deficiency on early transplant rejection after engrafting Ndst1−/− kidneys into WT Ndst1-expressing (Ndst1+/+) recipient mice. Effects of Ndst1 deficiency on donor solid organ renal allografts were compared to systemic blockade of chemokine-to-GAG interactions by M-T7 in WT renal allografts. Histopathological analysis of acute kidney allograft rejection was correlated with altered tissue immune cell invasion and with gene expression, chemokines, HS and CS GAG, and tissue disaccharides.

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Results

Ndst1 deficiency in donor renal allografts significantly reduces histopathological markers for early renal allograft rejection. As an initial assessment, the effects of conditional Tek/Tie2 promoter–driven Ndst1 deficiency in donor allografts on early rejection was examined in engrafted mice. Histopathological markers for early rejection in grafts with Ndst1 deficiency were compared to wildtype donor allografts, both treated with saline, at 10 days follow up (Fig. 1A,C,D–I). No other immune modulators were given. Histopathologic sections were read by pathologists blinded to donor organ, demonstrating significantly reduced rejection scores in Ndst1−/− and M-T7 treated WT compared to saline-treated WT allografts (Fig. 1D–J). Bar graphs also demonstrate significant decreases in individual pathologic parameter scores measured in Ndst1−/− donors compared to WT donors and for M-T7 treated WT allografts for Infiltrate (E), Vasculitis (F), Glomerulitis (G), Peritubular capillaritis (H), Tubulitis (I), and Mesangial matrix (J). P-value < 0.05 considered significant.

M-T7 treatment significantly reduces histopathological markers for renal allograft rejection. Chemokines interact with both GAGs and cell receptors and are reported to alter transplant rejection, with potential effect on donor organ endothelial glycocalyx, reduced early allograft and vascular inflammation and rejection.
Figure 2. M-T7 variants in wildtype C57Bl/6 (B6, WT) or Ndst1<sup>−/−</sup> transplants into Balb/C mice. SWISS Model visualization of M-T7 indicating the location of the E209I, R171E and F137D point mutations (A). Bar graphs indicating Overall Pathology of Rejection (B), Infiltrate (C), Vasculitis (D), Glomerulitis (E), Peritubular Capillaritis (F), Tubulitis (G), and Mesangial Matrix (H) scores for saline treated or M-T7 variant treated wildtype C57Bl/6 (white bars) or Ndst1<sup>−/−</sup> (gray bars) donor tissue. Numerical P-values above bars represent comparisons between wildtype and Ndst1<sup>−/−</sup> grafts for a specific treatment (adjacent bars). Asterisks indicate statistical comparison between saline and M-T7 or M-T7 variant for wildtype donor tissue only (white bars only) with *P* < 0.05, **P* < 0.01 and ***P* < 0.001.
with the broad-spectrum chemokine-GAG inhibitor M-T7 was examined in WT renal allograft transplants for comparison to Ndst1−/− donor allografts. Independent, blinded pathohistological analysis demonstrated significant reductions in histological markers of early allograft rejection at 10 days follow up with M-T7 treatment (10 daily doses, 100 ng/gm body weight) (Fig. 1B,D–I) in WT donor kidney transplants when compared to WT donor allografts treated with saline control (Fig. 1A). Changes produced by M-T7 were comparable to those seen with saline treatment of Ndst1−/− donor allografts implanted into BALB/c recipient mice. M-T7 reduced overall pathology scores for early rejection (Fig. 1D; P < 0.009) with independent reductions in cell infiltrates (Fig. 1E; P < 0.001), vasculitis (Fig. 1F; P < 0.036), glomerulitis (Fig. 1G; P < 0.0001), peritubular capillaritis (Fig. 1H; P < 0.013), and tubulitis (Fig. 1I; P < 0.0001). M-T7 did not reduce the score for mesangial matrix (Fig. 1J; P = 0.241), although treatment did indicate a trend toward reduction.

**M-T7 and M-T7 point mutant treatment have variable efficacy in WT and Ndst1−/− allografts.** M-T7-mediated reductions in markers of inflammation and early rejection were lost in Ndst1−/− donor transplants (Fig. 2, N = 32 mice). This is consistent with interference with the known M-T7-mediated inhibition of chemokine to GAG binding. However, the independent beneficial effects of both Ndst1 deficiency and M-T7 treatment on reducing inflammation and rejection in renal allografts were lost in combination (i.e., M-T7 treatment in Ndst1−/− donor organs) when compared to WT allografts (Fig. 2B–H).

To further define interactions between M-T7, HS-GAG and chemokines, three M-T7 point mutations, with previously characterized variations in chemokine and GAG binding (Fig. 2A), were assessed for altered effects on rejection in WT C57Bl/6 and also Ndst1−/− renal allograft transplants into BALB/c mice. Treatment with the M-T7 point mutations, F137D, R171E and E209I displayed differing inhibitory activities after transplant of C57Bl/6 WT donor kidneys into Balb/c mice (Fig. 2B–H). R171E (P < 0.001) and E209I (P < 0.01) retained significant inhibitory function in donor WT renal allografts, whereas F137D (P = 0.1774) no longer blocked early rejection (Fig. 2B–H).

R171E had no inhibitory activity in Ndst1−/− donor transplants, as was seen for M-T7 (Fig. 2). R171E therefore had minimal differences from the native M-T7 inhibitory activity in this model. Conversely, E209I retained inhibitory activity in both WT C57Bl/6 and Ndst1−/− donor renal allograft implants at 10 days follow up suggesting that E209I-mediated blockade of early signs of rejection is independent of Ndst1 or HS-GAG mediated chemokine interactions. F137D was inactive in both WT and in Ndst1−/− allografts. In prior work, AlphaScreen assays for R171E and E209I demonstrated reduced binding to the chemokine RANTES when compared to M-T7 and F137D (Fig. 2B–H). While all three tested point mutations had reduced RANTES binding in vitro, this effect was reduced for R171E and E209I. E209I had the smallest change in binding in the presence of heparin, suggesting that E209I may be less affected by heparin interaction with the chemokine–RANTES binding. Both R171E and E209I retained inhibitory activity for PMA-activated THP-1 cell migration in vitro, while F137D did not. There were also variations in independent histopathology findings for each mutant. F137D had an increase in vasculitis score (Fig. 2D) in Ndst1−/− donor allografts while E209I had a greater reduction in peritubular capillaritis in Ndst1−/− allografts (Fig. 2F).

**Reduced early rejection is associated with modified macrophage and T cell invasion.** Selective changes in macrophage and T cell tissue invasion were assessed in Ndst1−/− donor grafts and in M-T7 treated WT donor grafts with comparison to saline treated WT graft controls. Immunohistochemical analysis (Fig. 3) of Ndst1−/− allografts as well as M-T7 treated WT allografts demonstrated significant reductions in CD3+ T cells (Fig. 3A,G–E). In contrast F4/80 macrophage cell counts were reduced for saline treated Ndst1−/− allografts, but increased in M-T7 treated WT donors (Fig. 3B,F–H).

**Reduced HS and chemokine immunoreactivity is associated with reduced rejection.** Transplanted sections were examined using immunohistochemical staining for HS and chemokines (Fig. 4). Glomerular HS staining in saline-treated Ndst1−/− allografts was reduced when compared to saline treated WT transplant (P = 0.005; Fig. 4A–C), consistent with prior reports on Ndst1−/− tissue samples. M-T7 treatment demonstrated only a trend toward reduced HS staining (P = 0.063; Fig. 4C). When chemokine content was assessed, CXCL8 staining was significantly reduced in Ndst1−/− grafts and in M-T7 treated WT grafts (ANOVA P < 0.012; Fig. 4D,E). CC chemokine MCP-1 was not significantly reduced in either Ndst1−/− grafts or in M-T7 treated WT allografts (Fig. 4F,G).

**Altered gene expression in Ndst1−/− allografts and after M-T7 treatment in WT allografts.** Changes in gene expression in inflammatory and apoptotic pathways were measured in transplanted organs (Fig. 5). Significantly altered expression was detected for a subset of genes in signaling pathways as detected by qPCR analysis. M-T7-treated C57Bl/6 WT and Ndst1−/− donor tissue (both showing reduced rejection) was compared to saline-treated C57Bl/6 WT donors (Fig. 5A). Changes due to treatment with M-T7 and F137D mutant (no reduction in rejection) versus saline-treated C57Bl/6 WT donors was also compared (Fig. 5C). Among the detected gene expression changes, Interleukin 4 (IL-4) was significantly decreased for both Ndst1−/− and M-T7 treated WT grafts at 10 days follow up (Fig. 5A,B) versus saline-treated WT allografts. Heat shock transcription factor 1 (HSF1), Peroxisome proliferator-activated receptor gamma (PPARG), Telomerase reverse transcriptase (TERT), and WNT1 inducible signaling pathway protein 1 (WISP1) were significantly down-regulated in Ndst1−/− grafts, but not M-T7 treated WT allografts. MDMA2, CSF2, FOXA2, and TNF were significantly increased for M-T7 treated grafts, but not Ndst1−/− grafts. Whereas Nitric oxide synthase 2 (NOS2), TRAF family member-associated NfκB activator (TANK), Early growth response 1 (EGR1), Fibronecin 1 (FN1), CC chemokine CCL20, Heat shock protein 90AA2 (HSP90AA2), IGFBP3, Selectin E (SELE) were decreased in M-T7 treated WT allografts (Fig. 5A).
Specific gene expression changes were all within the NF\(\kappa\)B and JAK/STAT pathways, but with changes primarily selective for either Ndst1-deficient grafts or M-T7 treatment in WT grafts. In the NF\(\kappa\)B pathway, CCL20 was reduced by M-T7. In the JAK/STAT pathway, Interleukin-4 (IL-4) was significantly reduced in Ndst1\(^{-/-}\) grafts with saline treatment or in WT allografts with M-T7 treatment. NOS2 was also significantly reduced with M-T7 treatments in WT allografts. Although Murine double minute 2 (MDM2), a p53 regulator, was markedly increased by M-T7, this gene was also increased with F137D (Fig. 5C) treatment, which does not reduce rejection, suggesting a poor correlation with reduced rejection.

In summary, a series of genes in inflammatory signaling pathways demonstrated altered expression in grafts with reduced rejection. Significantly reduced IL-4 gene expression was detected for both Ndst1\(^{-/-}\) allografts and for M-T7 treated WT allografts (Fig. 5B). Significant changes for other genes differed in Ndst1\(^{-/-}\) grafts when compared to M-T7 treatment in WT grafts, suggesting differing targets.

### Altered HS and CS disaccharide content is detected in renal allografts with reduced rejection.
Altered GAG content and metabolism was also examined for correlations with graft rejection. HS and CS disaccharide content and sulfation were measured in isolates from Ndst1\(^{-/-}\) allografts and from M-T7 or saline-treated WT allografts. Kidney samples vary in weight and thus disaccharides were normalized to total HS or CS

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**Figure 3.** Immunohistological analysis of M-T7 treated WT and saline treated Ndst1\(^{-/-}\) donor renal allografts at 10 days post-transplant (N = 26 mice). Mean numbers of positively stained cells in 3 high power fields (HPF) are presented as bar graphs demonstrate significant decreases in CD3+ T cell infiltrates for saline treated Ndst1\(^{-/-}\) donors and for M-T7 treated WT donors (A). F4/80 stained monocytes were significantly decreased in saline treated Ndst1\(^{-/-}\) donors but significantly increased in M-T7 treated WT donors (B). Representative CD3+ micrographs (100X) for immunohistochemical CD3+ stained grafts, saline treated WT (C), saline treated Ndst1\(^{-/-}\) (D) and M-T7 treated WT (E). Immunohistochemical F4/80 stained graft micrographs for saline-treated WT (F), saline treated Ndst1\(^{-/-}\) (G) and M-T7 treated WT (H). Arrows indicate positively stained cells. P-value < 0.05 considered significant.
content or specimen weight, providing fractional disaccharide content (percentage weight). Total 2-O, 6-O and N-sulfation in disaccharides were also calculated. Overall N-sulfation (sum of D0S0, D0S6, D2S0 and D2S6 content), 6-O-sulfation (sum of D0A6, D0S6, D2A6 and D2S6) and 2-O-sulfation (sum of D2A0, D2S0, D2A6 and D2S6) as a percentage of total HS was calculated (Fig. 6). Overall N-sulfation in HS extracts was not altered (Fig. 6A). 6-O-sulfation in HS was significantly and unexpectedly increased in M-T7 treated WT transplants and saline treated Ndst1−/− transplants compared to controls (Fig. 6B; P < 0.0003 and 0.012, respectively). M-T7 also significantly increased percent 6-O-sulfation and 2-O-sulfation of CS, whereas Ndst1−/− donors did not show an increase when compared to WT saline treatment (Fig. 6D–F).

Ndst1−/− kidneys and M-T7 treated WT kidneys additionally had specific changes in percentage weight HS disaccharide, when compared to saline treated WT kidneys (Fig. 7). The percent weight (µg) of D0S6 was significantly increased in saline treated Ndst1−/− (P < 0.022) and in M-T7 treated WT transplants and saline treated Ndst1−/− transplants compared to controls (Fig. 6B; P < 0.0003 and 0.012, respectively). M-T7 also significantly increased percent 6-O-sulfation and 2-O-sulfation of CS, whereas Ndst1−/− donors did not show an increase when compared to WT saline treatment (Fig. 6D–F).

Figure 4. Immunohistochemical analysis of HS and chemokines. (A) Glomerular HS staining in saline treated WT allograft. (B) Reduced glomerular HS staining in Ndst1−/− allografts. (C) Bar graph demonstrates significant decrease in HS staining in saline-treated Ndst1−/− with only a trend in M-T7-treated WT renal allografts. (D) Bar graph demonstrates reduced IL-8 staining in Ndst1−/− allografts and in M-T7 treated WT allografts or Saline treated Ndst1−/− allografts. (E) IL-8 staining in WT allografts compared to Ndst1−/− or M-T7 treated allograft glomeruli. (F) Bar graph demonstrating no significant decrease in MCP-1 staining in Ndst1−/− or with M-T7 treatment of WT renal allografts. (G) MCP-1 staining in WT allografts compared to Ndst1−/− or M-T7 treated allograft glomeruli. Mag 100X.
compared to M-T7 treatment in WT grafts). As the method for labeling disaccharides can be complex, an explanatory diagram for HS disaccharide labeling is provided in Supplementary Fig. S1.

Significant changes were also observed in CS percent weights or CS disaccharides, although Ndst1 is reportedly selective for HS modification (Fig. 8). D0a4 (Fig. 8A) and D2a4 (Fig. 8B) CS disaccharides were reduced, D0a4 for both Ndst1−/− transplants and M-T7 treated WT transplants (ANOVA P < 0.0043) and borderline for D2a4 (ANOVA P = 0.1390). Total CS content (Fig. 8I) was significantly reduced in M-T7 treated renal transplants (P < 0.019), but not in Ndst1−/− transplants (ANOVA P = 0.078). Supplemental Figs S2 and S3 provide the HS and CS disaccharide data measurements using the same Y axis scale to allow for comparison of overall changes in content.

Combined changes in measured individual HS and CS disaccharides were correlated with overall pathology rejection scores measured on the same histology sections in Ndst1−/− or M-T7 treated WT kidneys by multiple linear regression analysis (for HS disaccharides R = 0.992, R2 = 0.984, for CS disaccharides R = 0.974, R2 = 0.949).

**Discussion**

Early and ongoing activation of inflammatory immune cell responses, also termed innate or acute cellular rejection, are reported to induce ongoing organ damage and to be a significant driving force for late chronic transplant vasculitis, rejection and graft loss. Late organ damage is also known to be caused by recurrent antibody-mediated immune rejection. Both antibody-mediated rejection and inflammatory cell responses are
reported to contribute approximately fifty percent to ongoing chronic rejection, graft damage and vasculopathy, occurring concomitantly in 25% or more of rejection episodes.

The result of this study on early rejection of renal allograft transplant demonstrates significant reductions in rejection after implant of donor kidneys deficient in Ndst1, the primary sulfotransferase HS-modifying enzyme (Fig. 1). Recipient BALB/c mice in this study have normal (wildtype) Ndst1 expression. Thus, the reduction in early rejection histopathology scores after transplant of Ndst1−/− donor organs with saline treatment is unique to the Ndst1-deficient donor organ, as no other treatment was given. In prior work, we demonstrated reduced aortic allograft inflammation and vasculopathy at later follow up times (4 weeks) in Ndst1−/− donor aortic grafts and after M-T7 treatment in WT aortic allografts 19. M-T7 also reduced chronic rejection and improved outcome in renal grafts at long term follow up in mice (100 days) and rats (5 months), respectively 19,26, but was not previously tested for effects on early or acute rejection. The aortic transplant model is considered a model for chronic transplant vasculopathy, more closely representative of chronic arterial inflammation and repair rather than antibody-mediated rejection 43,44. Thus, Ndst1 deficiency in the donor organ alone reduces both late (i.e., chronic) vasculopathy in aortic allografts and early or acute rejection in renal allografts. Significant and comparable reductions in rejection were also seen after treatment with M-T7, a broad-spectrum chemokine modulating protein that interferes with chemokine-GAG binding (Fig. 1). The capacity to modify rejection by altering GAG composition in the donor allograft may have broad potential for new treatment approaches in transplantation through modifying the donor organ.

While prior work has demonstrated that chemokines have an important role in immune and inflammatory responses in transplants, the role of the endothelial glyocalyx in donor organs has been less extensively studied 3,19,36–43,45. The capacity of an isolated decrease in Ndst1 expression specifically in the donor organ to significantly reduce early rejection suggests a central role for donor organ HS GAG content in rejection. Further the Ndst1 deficiency is selective for endothelial cells and myeloid precursors and one might predict that changes in donor Ndst1-deficient organs are predominately due to endothelial deficiency rather than myeloid precursors, as observed for thioglycollate-induced peritonitis and allergic contact dermatitis 14. Because all donor immune cells could not be removed prior to renal transplantation, we cannot exclude the possibility of hematopoietic microchimerism and resident suppression as an involved mechanism in our model 16–18. Indeed, it is known that LysM (leukocyte)-specific deletion of Ndst1 can affect inflammatory responses 49. Thus, there is a possibility that resident immune cells in the graft, which may be devoid of Ndst1, may play a role in the maintenance of graft integrity. Wang et al. performed control experiments on bone marrow chimeras to note that the predominant inflammation-associated Ndst1 knockout effects in this specific strain of mice are almost exclusively due to

![Figure 6](https://www.nature.com/scientificreports/)
knockout in the endothelium\textsuperscript{14}. Nevertheless, this remains to be proven in a transplant model and will require further investigation in future studies.

A significant reduction was detected in HS staining and IL-8 CXC chemokine staining with \textit{Ndst1}\textsuperscript{−/−} grafts suggesting that modified HS sulfation can interfere with chemokine-GAG gradient formation. However, M-T7 treatment, while reducing glomerular IL-8 CXC chemokine staining, did not significantly alter HS or CC MCP-1 chemokine staining (Fig. 4; $P = 0.063$).

We assessed changes in HS and CS content in renal allografts, but unexpectedly found increases in select HS disaccharides suggesting that \textit{Ndst1}\textsuperscript{−/−} deficiency reduced HS sulfation in the endothelium, but led to an increase in disaccharides in the whole transplanted organ, potentially due to a response in the graft to the local endothelial changes. There were shared differences in disaccharide content and sulfation for \textit{Ndst1}\textsuperscript{−/−} allografts and M-T7 treated WT allografts. HS disaccharide D0S6 was significantly increased for both saline treated \textit{Ndst1}\textsuperscript{−/−} and M-T7 treated WT kidneys (A), but D2S6 is only significantly increased in M-T7 treated WT grafts (B). D2A0 is reduced with M-T7 treatment (C). Total HS was not significantly altered (I) ($*P \leq 0.05$).

**Figure 7.** Bar graphs demonstrating changes in disaccharide content, measured as weight per weight fractions of total HS extracts, from saline treated \textit{Ndst1}\textsuperscript{−/−} and saline or M-T7 treated WT mouse kidneys after 10 days treatment. HS Disaccharide analysis for D0S6 (A), D2S6 (B), D2A0 (C), D0A6 (D), D0S0 (E), D2S0 (F), D2A6 (G), D0A0 (H) and Total HS (I). D0S6 is increased for both saline treated \textit{Ndst1}\textsuperscript{−/−} and M-T7 treated WT kidneys (A), but D2S6 is only significantly increased in M-T7 treated WT grafts (B). D2A0 is reduced with M-T7 treatment (C). Total HS was not significantly altered (I) ($*P \leq 0.05$).
To assess whether an overall change in disaccharide content might correlate with the risk of rejection, MR analysis was performed. A correlation between disaccharide content and rejection scores was detected, again suggesting a correlation between overall GAG content and rejection. Increased 6-O heparan sulfation has been previously reported in renal transplant biopsies with increased chronic fibrosis and rejection, thus indicating that altered D056 disaccharide content may represent one potential response to allograft rejection, whether protective or damaging. Conversely, sites of low sulfation have been associated with potentially inflammatory endoglycoside heparanase degradation of the glycocalyx and thus increased 6-O HS sulfation observed in our study (Fig. 6B) may be protective. Selective changes in disaccharide content may also be specific to individual cells as mast cell responses have demonstrated altered HS content with Ndst1 deficiency. Studies reported by other groups do support a pro-inflammatory function for some GAGs. Heparanase treatment in a donor stem cell transplant model is reported to reduce rejection, improve cell survival and reduce TGFβ2 responses and prevent diabetes in mice. HS is up-regulated in transplant vasculopathy (TAV) in chronic rejection, as well as in ischemia reperfusion injury. Antibodies to selected GAG species are reported to increase rejection. Conversely, low molecular weight heparin infusion reduces scarring as well as transforming growth factor (TGF) and collagen expression after renal obstruction injury in mice. Further work will be required to determine the specific interactions between transplant alone and each treatment in WT and in Ndst1 deficient transplants. Due to the complexity of GAG synthesis and modification, we note that these findings do not demonstrate a direct cause and effect between altered disaccharide composition in donor organs with early rejection, but rather highlight a consistent correlation requiring further study. HS GAGs have multiple functions in addition to...
chemokine-mediated cell activation and migration and further in-depth analyses will be required to determine
the exact mechanism(s) by which acute cell or antibody-mediated rejection is reduced with either Ndst1 defici-
cy or M-T7 treatment of allografts. We also report here a reduced efficacy for the M-T7 F137D point mutation
in treating WT kidney grafts, suggesting that the beneficial effect of M-T7 in acute rejection with WT donors is
specific to M-T7 mediated inhibition of chemokine binding to GAG alone and not through direct M-T7 interac-
tion with GAG. The F137D mutant is predicted to have reduced chemokine interaction when compared to native
M-T7 as the hydrophobic region of the structure that interacts with chemokines is disrupted. This supports the
hypothesis that M-T7 reduces transplant rejection via inhibition of chemokines. In prior work F137D had reduced
inhibition of chemokine binding when treated with heparin35. However, the finding here for F137D, differed
from prior findings examining plaque growth in a mouse model of balloon angioplasty injury in hyperlipidemic
Apoe−/− mice, where F137D retained some inhibitory activity, but did mirror the loss of inhibition for PMA acti-
vation on monocytes in vitro36. E209I was less affected by heparin competition in prior work in vitro37, and retained
activity in Ndst1−/− allografts in the current study. These observations suggest the possibility that E209I protein
functions independent of GAG interactions. This difference in responsiveness for balloon angioplasty injury and
solid organ transplant is not unexpected, as mechanisms underlying acute organ transplant rejection differ from
those driving plaque growth after simple mechanical balloon angioplasty injury where there is no rejection.
Selective reduction in transplant organ CD3+ T cell invasion correlated with reduced rejection. The individ-
ual changes in modification of inflammatory response pathway genes support a key role for modification of
innate and acquired immune responses in rejection, both via Ndst1 deficiency and with M-T7 treatment. The
reduction in IL-4 gene expression by both approaches, M-T7 treatment in WT allografts and in saline treated
Ndst1−/− allografts, does suggest again shared or convergent regulatory pathways for Ndst1−/− donor grafts and
for M-T7 treatment in WT grafts. While the change in MDM2 is large and may represent a significant regulatory
step, there is no comparable increase in Ndst1−/− kidney and treatment with the inactive F137D point mutation
also increased this gene, making this change of lesser interest. A transcriptome-wide analysis for genes that corre-
late with histopathological changes in rejection would be preferable, and will be approached in future analyses34.
These findings correlate the reduced rejection observed in saline treated Ndst1−/− engrafted mice to altered HS
and CS content, potentially via blockade of chemokine interactions. The observed reduction in overall rejection
score is very similar to systemic treatment with M-T7, a broad-spectrum inhibitor of chemokine-GAG inter-
actions. However, modulation of other GAG-dependent functions has yet to be examined in this model, and
immune modulators other than chemokine-GAG interactions may play key functions. Further work will be nec-
essary to examine other potential HS GAG interactions modified by Ndst1 deficiency or M-T7 treatment.

Conclusions
In conclusion, reducing endothelial and myeloid precursor cell Ndst1 expression, in donor allograft transplants
alone, reduces acute renal allograft rejection, comparable to chemokine inhibition. Changes in donor organ HS
disaccharide composition in transplanted organs has potential for diagnosis in detecting early rejection. These
findings may also guide new donor-focused approaches to treating transplant organs designed to reduce acute
inflammation and prevent chronic allograft damage, which is an unmet therapeutic need.

Materials and Methods
Animals. Three strains of mice were used in this study. C57BL6/J (stock #000664) and BALB/c (stock
#000651) mice were obtained from JAX Laboratories (Bar Harbor, MN) or the University of Florida Animal
Care Services breeding facility, which replenishes stocks from JAX Laboratories. Derivation and characteri-
ization of Ndst1TekCre+ mice (Ndst1−/−); kindly provided by Dr. Jeffrey D. Esko, Glycobiology Research and
Training Center, University of California, San Diego, CA) with floxed Ndst1 conditionally knocked out by Tek/
Tie2 endothelial tyrosine kinase promoter-driven Cre in the endothelial and sub-population leukocytes have
been previously described14. All animals were housed in barrier conditions in vivaria of the University of Florida
Animal Care Services. Mice were weaned at 3 weeks, maintained on a 12-hour light–dark cycle and were fed
water and standard rodent chow ad libitum.

Surgical protocols - Kidney transplantation. All animal studies complied with University and National
Institutes of Health guidelines for the care and use of Laboratory animals and were approved by the University
of Florida (UFL) and Arizona State University (ASU) Institutional Animal Care and Use committee (IACUC; UFL
IACUC Protocol # 201604234_01; ASU IACUC Protocol #17-1549R). Renal allograft transplant was per-
formed as previously described (Table 1; 6–10 mice with allograft transplant per donor organ genetic strain and
treatment type; Total 80 mice). In brief, the donor kidney is placed in the left flank in the mouse and attached by
end-to-side anastomosis between the donor suprarenal aortic cuff and the recipient aorta. Venous anastomosis
between donor suprarenal inferior vena cava (IVC) and recipient IVC is performed in the same fashion and the
bladder attached, as previously described19. A series of donor renal allografts from either C57BL/6 wild type (WT) or
Ndst1−/− were transplanted into BALB/c mice. Mice with WT or Ndst1−/− donor allografts were treated with either saline control, M-T7 or indi-
vidual mutated constructs (M-T7-His6X, F137D, R271E, or E209I; 6–10 mice per donor organ strain and per treat-
ment) (Table 1)39,44,52. Donor renal allografts were transplanted into BALB/c mice after resection of both kidneys
under general anesthetic. No other immune suppressants were given to the mice before or after transplantation, in
order to examine isolated early effects of each condition alone on transplant rejection at 10 days follow up31–33. Treat-
ments were given daily by intraperitoneal (IP) injection at 100 ng/gm/day X 10 days per mouse for each
individual protein treatment19. Mice were euthanized at 10 days follow up with Euthanyl (Virbac AH Inc., Fort
Worth, TX) as previously described19. For tissue analyses, renal allografts were divided into 3 sections and each
section cut in half; one third fixed in 10% neutral-buffered formalin for histology and the other two thirds cut in half and stored frozen or stored in RNAlater for RNA analysis.

**M-T7 and M-T7 point mutation generation and expression.** M-T7 and M-T7 point mutations were expressed and purified as previously described. In brief, M-T7 mutants were generated by mutagenic PCR using M-T7pFastBacDualeGFP as the template. Mutant constructs and wild type M-T7 were transformed into DH10Bac bacteria (Invitrogen, Carlsbad, CA), and blue/white screened on LB + Kan + Tet + Gen + IPTG + X-gal plates. Bacmids were purified and used to transfect S9 insect cells with Cellfectin II (Invitrogen, Carlsbad, California). Baculovirus supernatants were collected to infect insect cells and express the various M-T7 mutant proteins. M-T7 and each of the three mutant constructs were then purified by sequential column purification as previously described.

**Histological and immunohistochemical analysis of acute rejection and scarring.** Sections of transplanted organs were cut into three 1.5–2 mm equal length cross sections for histology, fixed, paraffin embedded, and cut into 4–5 μm sections (3 sections per transplant specimen, providing 9 sections per allo-graft). Histology sections were stained with Haematoxylin and Eosin (H & E), Masson’s trichrome, and Periodic acid-Schiff (PAS) as previously described. All sections were analyzed for changes consistent with acute rejection and vasculitis by pathologists blinded to the mouse donor allograft implant and to treatment with either saline or M-T7 using Banff diagnostic criteria (DW, WC, BC). Pathology was scored on a scale of 4 and overall pathology score was a summation of independent scores assessed by detection of cellular infiltrate, vascu-litis, glomerulitis, peritubular capillaritis, tubulitis, and mesangial matrix.

Renal allografts were assessed by immunohistochemical staining for macrophage and T lymphocyte invasion and for HS with rabbit polyclonal against F4/80 (macrophages; ab100790 from Abcam at 1:50 dilution), rabbit polyclonal against CD3 (T cells; ab5690 from Abcam at 1:50 dilution) and mouse antibody to 10E4 epitope of HS and for HS with rabbit polyclonal against F4/80 (macrophages; ab100790 from Abcam at 1:50 dilution), rabbit polyclonal against CD3 (T cells; ab5690 from Abcam at 1:50 dilution), and mouse antibody to 10E4 epitope of HS (Clone F58–10E4 from AMSBio at 1:50 dilution) using ABC staining technique, respectively and as previously described. Yyclonal against CD3 (T cells; ab5690 from Abcam at 1:50 dilution) and mouse antibody to 10E4 epitope of HS and for HS with rabbit polyclonal against F4/80 (macrophages; ab100790 from Abcam at 1:50 dilution), rabbit polyclonal against CD3 (T cells; ab5690 from Abcam at 1:50 dilution), and mouse antibody to 10E4 epitope of HS (Clone F58–10E4 from AMSBio at 1:50 dilution) using ABC staining technique, respectively and as previously described.

**RT-PCR array analysis of altered gene expression in renal allografts.** One third of each transplanted kidney section was collected in RNAlater (Ambion, Austin, TX) and RNA was isolated using RNaseasy Mini kit following the manufacturer’s protocol (Qiagen, Valencia, CA). RNA was reverse transcribed to cDNA using Superscript VILO cDNA Synthesis kit (Invitrogen Corporation, Carlsbad, CA) and Real Time PCR carried out using SYBR Green Core Reagent kit and a 7300 RT-PCR system (Applied Biosystems, Austin, TX). Changes in gene expression were normalized to internal GAPDH control and subsequently to saline treated controls. Primers specific to inflammatory and apoptotic pathways were assayed and are listed in Supplemental Table 1.

**Analysis of GAGs from saline treated Ndst1−/− and WT kidneys, with and without M-T7.** Total HS- and CS-GAG content and percent weight disaccharide were measured in transplanted kidneys from WT mice, with and without M-T7 treatment, and in transplanted kidneys from Ndst1−/− mice with saline or M-T7 treatment (N = 10, 3–4 mice per strain and treatment group). Researchers were blinded to samples (SA, PA). HS and CS GAG composition were quantified by HPLC. Formalin-fixed, paraffin-embedded samples were extracted as previously described. Samples were incubated in glass tubes in a heating block at 60°C with 3 mL of

| Donor | Recipient | Treatment | Number mice | Follow up (days) | Survival |
|-------|-----------|-----------|-------------|-----------------|----------|
| C57Bl/6 | Balb/C | Saline | 10 | 10 | 10/10 |
| NDST1−/− | Balb/C | Saline | 9 | 9 | 9/9 |
| C57Bl/6 | Balb/C | M-T7 (100 ng/gm) | 7 | 10 | 7/7 |
| NDST1−/− | Balb/C | M-T7 (100 ng/gm) | 6 | 6 | 6/6 |
| C57Bl/6 | Balb/C | R171E (100 ng/gm) | 10 | 10 | 10/10 |
| NDST1−/− | Balb/C | R171E (100 ng/gm) | 6 | 6 | 6/6 |
| C57Bl/6 | Balb/C | F137D (100 ng/gm) | 10 | 10 | 10/10 |
| NDST1−/− | Balb/C | F137D (100 ng/gm) | 6 | 6 | 6/6 |
| C57Bl/6 | Balb/C | E209I (100 ng/gm) | 10 | 10 | 10/10 |
| NDST1−/− | Balb/C | E209I (100 ng/gm) | 6 | 6 | 6/6 |
| Total numbers of mouse renal allografts | | | 80 | 80 | 80/80 (100%) |
xylene, heated for 15 minutes with 2 repeats, and then rinsed with 3 mL of 100% ethanol, followed by 96, 70, 50, and 30% ethanol in water and washed three times with 18 MΩ water. Samples were rehydrated in 1 mL solution of PBS for >30 minutes at RT. Rehydrated tissue was homogenized and defatted in acetone over 48 h with shaking at 4 °C. Samples were dried and suspended in 2 mL of 0.1 M Tris-HCl, pH 8.0, containing 2 mM CaCl₂, and 1% Triton X-100. Pronase (0.8 mg/mL) was added to the suspension and tissue digested at 50 °C for 24 h with one 24 h repeat. Finally, pronase was inactivated by heating to 100 °C for 15 min. Buffer was then adjusted to 2 mM MgCl₂, benzonase (100 mU) added, and samples incubated for 2 h at 37 °C. After inactivation of the enzyme (15 min, 100 °C), undigested tissue was removed by centrifugation for 1 h at 400 g.

Sample supernatant was applied to a DEAE-Sepharose – micro spin column (Harvard Apparatus), washed with ~10 column volumes (CVs) of loading buffer (~pH 8.0 Tris Buffer) twice, allowed to adhere, washed with ~20 CVs of loading buffer, followed by ~20 CVs of wash buffer (~pH 4.0 Acetate Buffer), and 3 CVs of water. Samples were further cleaned with ~15 CVs of 0.2 M NH₄HCO₃. To elute, ~20 CV of 2 M NH₄HCO₃ was added to the column and the fractions were collected. Samples were then freeze-dried and dissolved in 100 µL water.

Samples were digested with Heparinases I-III (New England Biolabs) or Chondroitinase ABC (Sigma), producing disaccharides that were separated using SAX-HPLC coupled to post-column fluorescence labeling and detection (Agilent system using a 4.6 × 250 mm Waters Spherisorb analytical column with 5 µm particle size at 25 °C). Peak migration times and areas were calculated compared to known disaccharide standards. Representative chromatograms are provided in Supplemental Fig. 4. HPLC was run with two solvents, Solvent A: 2.5 mM sodium phosphate, pH 3.5 and Solvent B: 2.5 mM sodium phosphate, 1.2 M NaCl, pH 3.5 with a gradient change from 97% A and 3% B to 100% B and 0% A (65 min, flow rate 1.0 mL/min). GAG detection was performed by post-column derivatization. Eluents were combined with a 1:1 mixture of 0.25% NaOH and 1% (w/v) 2-cyanoacetamide pumped at a flow rate of 0.5 mL/min from a binary HPLC pump (SSI Scientific Systems, Inc) and heated to 130 °C in a 10 min reaction coil, before cooling and return into the Agilent's fluorescence detector (λex = 346 nm, λem = 410 nm). Commercial standard disaccharides (Dextra Laboratories) were used for identification based on elution time, as well as for calibration.

Paraffin-containing formalin from each sample was assessed in parallel as a control. No GAG or disaccharide was detected in these controls. Following isolation the GAGs released with β-elimination (1% w/w sodium borohydride in 2N NaOH) were desalted with a PD-10 column (GE Healthcare), and freeze-dried before disaccharide analysis. Peak migration times and areas for disaccharides separated by HPLC were compared to known standards (See example in Supplemental Fig. S4).

**Statistical analysis.** Measured change in histopathology scores, incorporating tissue mononuclear cell count, percentage of positively stained cells, PCR array, HS staining, and tissue HS and CS disaccharide content were assessed for statistical significance using analysis of variance (ANOVA) with secondary Fisher’s PLSD or Student’s t-test. Multiple and simple regression analyses were performed to assess correlations between tissue disaccharide content with total histopathologic score for acute rejection.  

### References

1. Gottmann, U. et al. Influence of hypersulfated low molecular weight heparins on ischemia/reperfusion: Injury and allograft rejection in rat kidneys. *Transplant. Int.* **20** (2007).

2. Radio, S. et al. Allograft vascular disease: comparison of heart and other grafted organs. *Transplant. Proc.* **28**, 496–499 (1996).

3. Bagnasco, S. M. & Kraus, E. S. Intimal arteritis in renal allografts: new takes on an old lesion. *JAMA J. Am. Med. Assoc.* **278**, 1993 (1997).

4. Foros, E. & Kjellén, L. Heparan sulphate heparanases: Lessons from knockout mice. *J. Clinical Investigation* **105**, 175–180 (2000).

5. Kuchen, G. S. et al. Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry* **38**, 12959–12968 (1999).

6. Kuchen, G. S. et al. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 1885–1890 (2003).

7. Dai, E. et al. Inhibition of Chemokine-Glycosaminoglycan interactions in donor tissue reduces mouse allograft vasculopathy and transplant rejection. *PLoS One* **5**, e108 (2010).

8. Rops, A. L. et al. Modulation of heparan sulfate in the glomerular endothelial glycocalyx decreases leukocyte influx during experimental glomerulonephritis. *Kidney Int.* **86**, 932–942 (2014).

9. Mennander, A., Räisänen, A., Paavonen, T. & Häyry, P. Chronic rejection in the rat aortic allograft. V. Mechanism of the angiopeptin (BIM23014C) effect on the generation of allograft arteriosclerosis. *Transplantation* **55**, 124–8 (1993).
21. Miller, L. W. et al. Inhibition of transplant vasculopathy in a rat aortic allograft model after infusion of anti-inflammatory viral serpin. *Circulation* **101**, 1598–605 (2000).

22. Raines, E. W. The extracellular matrix can regulate vascular cell migration, proliferation, and survival: relationships to vascular disease. *Int. J. Exp. Pathol.* **81**, 173–82 (2000).

23. Lucas, A. & McFadden, G. Secreted Immunomodulatory Viral Proteins as Novel Biotherapeutics. *J. Immunol.* **173**, 4765–4774 (2004).

24. Lalani, A. S. et al. Functional comparisons among members of the poxvirus T135KDa family of soluble CC-chemokine inhibitor glycoproteins. *Virology* **250**, 173–184 (1998).

25. Upton, C., Mossman, K. & Mcfadden, G. Encoding of a Homolog of the IFN-γ Receptor by Myxoma Virus. *Science* (80-.). **258**, 1369–1372 (1992).

26. Bédard, E. L. R. et al. Chemokine-binding viral protein M-T7 prevents chronic rejection in rat renal allografts. *Transplantation* **76**, 249–252 (2003).

27. Liu, L. et al. Viral chemokine-binding proteins inhibit inflammatory responses and aortic allograft transplant vasculopathy in rat models. *Transplantation* **77**, 1652–1660 (2004).

28. Liu, L. et al. The viral anti-inflammatory chemokine-binding protein M-T7 reduces intimal hyperplasia after vascular injury. *J. Clin. Invest.* **105**, 1613–1621 (2000).

29. Bartee, M. Y. et al. Defining the anti-inflammatory activity of a potent myxoviral chemokine modulating protein, M-T7, through site directed mutagenesis. *Cytokine* **65**, 79–87 (2014).

30. Couto, R. M. et al. Structures of Orf Virus Chemokine Binding Protein in Complex with Host Chemokines Reveal Clues to Broad Binding Specificity. *Structure* **23**, 1199–1213 (2015).

31. Nesbitt, T., Coffman, T. M., Griffiths, R. & Drezner, M. K. Crosstransplantation of kidneys in normal and Hyp mice evidence that the Hyp mouse phenotype is unrelated to an intrinsic renal defect. *J. Clin. Invest.* **89**, 1453–1459 (1992).

32. Mannon, R. B. et al. Rejection of kidney allografts by MHC class I-deficient mice. *Transplantation* **59**, 746–755 (1995).

33. Cheng, O. et al. Connective tissue growth factor is a biomarker and mediator of kidney allograft fibrosis. *Am. J. Transplant.* **6**, 2292–2306 (2006).

34. Reeve, J. et al. Diagnosing rejection in renal transplants: A comparison of molecular- and histopathology-based approaches. *Am. J. Transplant.* **9**, 1802–1810 (2009).

35. Christov, A. et al. In vivo optical analysis of quantitative changes in collagen and elastin during arterial remodeling. *Photochem. Photobiol.* **81**, 457–466 (2005).

36. De Muro, P. et al. Kidney post-transplant monitoring of urinary glycosaminoglycans/ proteoglycans and monokine induced by IFN-γ (MIG). *Clin. Exp. Med.* **13**, 59–65 (2013).

37. Reitmaa, S., Saaf, D. W., Vink, H., Van Zandvoort, M. A. M. J. & Oude Egbrink, M. G. A. The endothelial glycolalyx: Composition, functions, and visualization. *Pflogers Archiv European Journal of Physiology* **454**, 345–359 (2007).

38. Rienstra, H. et al. Differential expression of proteoglycans in tissue remodeling and lymphangiogenesis after experimental renal transplantation in rats. *PLoS One* **5**, e9095 (2010).

39. Yard, B. A., Lorentz, C. P., Herr, D. & van der Woude, F. J. Sulfation-dependent down-regulation of interferon-gamma-induced major histocompatibility complex class I and II and intercellular adhesion molecule-1 expression on tubular and endothelial cells by glycosaminoglycans. *Transplantation* **66**, 1244–1250 (1998).

40. Celis, J. W. A. M. et al. Subendothelial heparan sulfate proteoglycans become major L-selectin and monocyte chemoattractant protein-1 ligands upon renal ischemia/reperfusion. *Am. J. Pathol.* **170**, 1865–1878 (2007).

41. Joosten, S. A. et al. Antibody response against perlecans and collagen types IV and VI in chronic renal allograft rejection in the rat. *Am. J. Pathol.* **160**, 1301–1310 (2002).

42. Alhasan, A. A., Spielhofer, J., Kusche-Gullberg, M., Kirby, J. A. & Ahl, S. Role of 6-O-sulfated heparan sulfate in chronic renal fibrosis. *J. Biol. Chem.* **289**, 20295–20306 (2014).

43. Libby, P. & Pober, J. S. Chronic rejection. *Immunity* **14**, 387–397 (2001).

44. Haney, P. Pathophysiology of chronic rejection. *Transplant. Proc.* **28**, 7–10 (1996).

45. Lawrence, R., Lu, H., Rosenberg, R. D., Esko, J. D. & Zhang, L. Disaccharide structure code for the easy representation of constituent oligosaccharides from glycosaminoglycans. *Nat. Methods* **5**, 291–292 (2008).

46. Larsen, C. P., Morris, P. J. & Austyn, J. M. Migration of dendritic leukocytes from cardiac allografts into host alveoli. A novel pathway for initiation of rejection. *J. Exp. Med.* **171**, 307–14 (1990).

47. Elwood, E. T. et al. Microchimerism and rejection in clinical transplantation. *Lancet* **349**, 1358–1360 (1997).

48. Ochando, J., Conde, P. & Bronte, V. Monocyte-Derived Suppressor Cells in Transplantation. *Curr. Transplant. Reports* **2**, 176–183 (2015).

49. Gords, P. L. S. M. et al. Reducing macrophage proteoglycan sulfation increases atherosclerosis and obesity through enhanced type i interferon signaling. *Cell Metab.* **20**, 813–826 (2014).

50. Naggi, A. et al. Modulation of the heparanase-inhibiting activity of heparin through selective desulfation. graded N-acetylation, and glycol splitting. *J. Biol. Chem.* **280**, 12103–12113 (2005).

51. Dagvöl, A., Holmborn, K., Kjellén, L. & Åbrink, M. Lowered expression of heparan sulfate/heparin biosynthesis enzyme N-deacetylase/N-sulfotransferase 1 results in increased sulfation of mast cell heparin. *J. Biol. Chem.* **286**, 44433–44440 (2011).

52. Chen, H. et al. Viral serpin therapeutics: From concept to clinic. *Methods Enzymol.* **499**, 301–329 (2011).

53. Pomin, V. H. et al. Exploiting enzyme specificities in digestions of chondroitin sulfates A and C: Production of well-defined heparans. *Glyobiology* **22**, 826–838 (2012).

54. Van Wijk, X. M. R. et al. Extraction and structural analysis of glycosaminoglycans from formalin-fixed, paraffin-embedded tissues. *Glyobiology* **22**, 1666–1672 (2012).

**Acknowledgements**

This work was supported by the National Institutes of Health (Grant number 1RC1HL10202-01), the American Heart Association (Grant numbers 0855421 E, 12GRNT120/0313, and 17GRNT3460327) and University of Florida Gatorade (Grant number 00115070) to A.L. This research was also supported in part by the National Institutes of Health (NIH)-funded Research Resource for Integrated Glycotechnology (NIH grant no. 5P41GM1039024) to P.A. at the Complex Carbohydrate Research Center. We would like to thank Dr. Jeffrey Esko (UCSD) for his advice and expertise in glycosaminoglycans and Dr. Barbara Munk for her help in proofing and revising this manuscript.

**Author Contributions**

Conceived experiments – H.C., A.L., A.P; Wrote and proofed manuscript – A.L., H.C., J.R.Y., L.Z., A.P., S.A., C.B., K.R., S.A.A.-H.; Performed experiments and analyzed data – H.C., S.A., J.R.Y., D.Z., A.L., M.B., S.M., D.W., W.C., M.B., S.A.A.-H., P.A.; Performed experiments – K.E., V.M.; Prepared figures – A.L., J.R.Y., S.A., D.Z., W.C., D.W.
Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-31779-7.

Competing Interests: For potential COI, we would note that Dr. A. Lucas has previously been affiliated with a small Biotechnology company in Canada, but that company was not involved in this research and is no longer functioning. Dr. Lucas has submitted patents on M-T7 and to Ndst1 modification with antisense oligonucleotides to Ndst1 in transplant. We do have older patents related to the chemokine modulating protein, M-T7 and have had active research grants funded by the American Heart Association as well as NIH funding.

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