Colitis, Hypothyroidism, and Immunological Alterations in Mice Deficient in Glycan Branching Enzymes.

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biology

by

Erica Lyn Stone

Committee in charge:

Professor Jamey D. Marth, Chair
Professor Stephen M. Hedrick, Co-Chair
Professor Steven F. Dowdy
Professor Ananda W. Goldrath
Professor Maho Niwa
The Dissertation of Erica Lyn Stone is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2009
To my brother,

David Charles Stone.

In recognition of all you taught me about life.
EPIGRAPH

Change will not come if we wait for some other person of some other time. We are the ones we've been waiting for. We are the change that we seek.

Barack Obama
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LIST OF ABBREVIATIONS

αDG: α-dystroglycan; ALT: alanine transaminase; AP: alkaline phosphatase; APRIL: A Proliferation-Inducing ligand; BSA: bovine serum albumin; C2GnT: Core 2 β1,6-N-acetylglicosaminyltransferase; C3GnT: Core 3 β-1,3-N-acetylglicosaminyltransferase; CCR7: C-C Chemokine Receptor 7; CD40L: CD40 ligand; CFA: Complete Freund’s Adjuvant; conA: concanavalin A; Core1-β3GnT: Core 1 Extension-β1,3-N-acetylglicosaminyltransferase; Core 1 GalT: Core 1 β-1,3-galactosyltransferase; CSFE: Carboxyfluorescein succinimidyl ester; CT: cholera toxin; DAI: disease activity index; DC: dendritic cell; DHB: 2,5-dihydroxybenzoic acid; DNP: 2,4-dinitrophenyl hapten; DSS: dextran sodium sulfate; ES: embryonic stem; FucT: α(1,3)fucosyltransferase; GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglicosamine; GALT: gut-associated lymphoid tissues; HEV: high endothelial venules; HRP: horseradish peroxidase; H&E: hematoxylin and eosin; IBD: Inflammatory Bowel Disease; IFA: Incomplete Freund’s Adjuvant; IFNγ: Interferon-gamma; IGnT: I β-1,6-N-acetylglicosaminyltransferase; IL: Interleukin; i.p.: intraperitoneal; KLH: keyhole limpet hemocyanin; MALDI: Matrix-assisted laser desorption/ionization; MS: mass spectrometry; MS/MS: tandem mass spectrometric; Muc2: Mucin 2; MHC Class II: major histocompatibility complex class II; NHS: N-hydroxysuccinimide; UC: ulcerative colitis; OVA: ovalbumin; PBS: phosphate buffered saline; PBST: PBS plus 0.05% Tween 20; ppGalNAcT: polypeptide N-α-acetylgalactosaminyltransferases; PNA: peanut
agglutinin; POMGnT1: protein-O-mannose-β1,2-N-acetylglucosaminytransferase 1; ST3Gal: α-2,3-sialyltransferase; T4: Thyroxine; tk: thymidine kinase; TMB: Tetramethylbenzidine; TNBS: 2,4,6-trinitrobenzene sulfonic acid; TRH: thyrotropin-releasing hormone; TSH: Thyroid Stimulating Hormone
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Chapter 2 in part contains data obtained through collaboration with Mohd Nazri Ismail, Stuart M. Haslam and Anne Dell to determine O-glycan structures. Chapter 2 also contains in part data and modified text from the abstract, background and significance, and preliminary studies sections of a
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CURRICULUM VITA

Education:

9/99-5/03 University of New Hampshire
Bachelor of Science in Biology: Molecular, Cellular and Developmental
Summa Cum Laude

9/03-3/09 University of California, San Diego
Doctor of Philosophy in Biology

Work Experience:

12/00-8/03 Intern, EMD Lexigen Research Center

Awards:

2003 University Honors-in-Major, University of New Hampshire

2002 and 2003 Presidential Scholar, University of New Hampshire

Publications:


Abstracts:


Teaching:

Molecular Biology. Winter, 2007
University of California, San Diego

AIDS in Society. Spring, 2006
University of California, San Diego

Biochemical Techniques Laboratory. Fall, 2004
University of California, San Diego

Principles of Biology I Laboratory. Fall 2001.
University of New Hampshire
ABSTRACT OF THE DISSERTATION
Colitis, Hypothyroidism, and Immunological Alterations in Mice Deficient in Glycan Branching Enzymes.

by

Erica Lyn Stone

Doctor of Philosophy in Biology
University of California, San Diego, 2009

Professor Jamey D. Marth, Chair
Professor Stephen M. Hedrick, Co-Chair

A majority of my dissertation research has focused on investigating why multiple glycosyltransferases with the ability to produce Core 2 O-glycans, the Core 2 β1,6-N-acetylglucosaminytltransferases (C2GnTs), have been conserved. For these studies I have utilized mice singly and multiply deficient in C2GnTs. Our results begin to explain why these three separate glycosyltransferases have been conserved, as each singly deficient strain exhibits distinct phenotypes. C2GnT1 has previously been shown to be required for normal selectin ligand formation. We found that absence of C2GnT2 results in reduced mucosal barrier function and increased disease pathogenesis following treatment with dextran sodium sulfate to induce colitis. C2GnT2-deficient mice also have reduced circulating and mucosal immunoglobulins, likely resulting from decreased antigen-specific humoral
responses to mucosal but not parenteral antigens. In contrast, mice deficient for C2GnT3 exhibited increased dominance in the tube test for social dominance linked to reduced Thyroxine abundance in circulation. Remarkably, we found that mice deficient for all three C2GnTs were viable and lacked all Core 2 O-glycan structures. Analysis of O-glycan structures present in the various singly deficient models revealed that all 3 C2GnTs contribute to Core 2 O-glycan biosynthesis \textit{in vivo}, albeit to differing degrees. We further found that loss of C2GnTs resulted in unexpected alterations in O-glycosylation, including increased abundance of elongated Core 1 O-glycans. Furthermore, elongated O-mannose structures were unexpectedly present in stomach tissue from C2GnT2-deficient mice or mice lacking all three C2GnTs.
GENERAL INTRODUCTION

Protein glycosylation is a common post-translation modification implicated in a wide range of biological processes including development, fertility, immune homeostasis, apoptosis, barrier function, trafficking, and microbe-host interactions (Lowe and Marth, 2003; Kawakubo et al., 2004; An et al., 2007; Marth and Grewal, 2008). Several types of protein glycosylation occur including O-linked and N-linked glycosylation of proteins in the secretory system and O-GlcNAcylation of cellular and nuclear proteins. My dissertation research focused on investigating the physiological effects of loss of one or more enzymes of the Core 2 β1,6-N-acetylglucosaminyltransferases (C2GnT) family of glycosyltransferases, which generate a highly abundant O-glycan type, termed Core 2 O-glycans.

The Biosynthesis of The Major Cores of Mucin-type O-glycans

Several O-glycan types are generated in the mammalian secretory system, and these types are defined based on the first monosaccharide transferred to the hydroxyl group. Mucin-type O-glycosylation, which is initiated with the transfer of a N-acetylgalactosamine (GalNAc), is the best studied, and thus often referred to simply as O-glycosylation (This simpler term will be used frequently in this dissertation to refer to Mucin-type O-glycosylation.) (Marth, 1999). Other types of O-linked glycans include O-
mannose, O-fucose, O-glucose, and proteoglycans, which are initiated when a xylose is added to the hydroxyl group of serines (Marth, 1999). Most of my graduate research focused on Mucin-type O-glycosylation, a common modification of the mucin glycoproteins. Mucins derive a majority of their weight from O-glycans and are the major component of mucus.

Mucin-type O-glycosylation is initiated in the golgi when one of many polypeptide N-α-acetylgalactosaminyltransferases (ppGalNAcT) transfers a GalNAc from UDP-GalNAc to the hydroxyl group of serine or threonine residues generating what is referred to as the Tn antigen (Figure I.1) (Ten Hagen et al., 2003; Tarp and Clausen, 2008). Various glycosyltransferases compete at different steps to elongate, branch, and decorate the glycan resulting in the generation of a diverse repertoire of O-glycans. This diverse repertoire of O-glycans can be grouped into several subtypes based on what is referred to as their core structure. The core structure refers to common non-sialic acid monosaccharides that are linked to the initiating GalNAc of O-glycans. There are 4 commonly occurring core subtypes in mammalian O-glycans, Core 1 through Core 4.

Commonly, Core 1 β-1,3-galactosyltransferase (Core 1 GalT) acts on the Tn antigen, adding a galactose in a β1,3-linkage to the GalNAc thereby generating the initial Core 1 O-disaccharide (also referred to as the T-antigen) (Ju et al., 2002). One of three Core 2 β1,6-N-acetylglicosaminyltransferases (C2GnT1, C2GnT2 and C2GnT3) can then act on the unmodified Core 1
structure transferring a \(N\)-acetylglucosamine (GlcNAc) from UDP-GlcNAc in a \(\beta\)1,6-linkage to the GalNAc residue generating the Core 2 branch of O-glycans (Figure I.1) (Bierhuizen and Fukuda, 1992; Schwientek et al., 1999; Yeh et al., 1999; Schwientek et al. 2000). Once the Core 2 branch is initiated the glycan is referred to as a Core 2 O-glycan to indicate that the O-glycan structure includes both a Core 1 branch and a Core 2 branch. The term Core 1 O-glycan is used to refer to O-glycan structures containing only the Core 1 branch.

In one alternative pathway, Core 3 \(\beta\)-1,3-\(N\)-acetylglucosaminyltransferase (C3GnT) can transfer a GlcNAc to the Tn antigen initiating the Core 3 branch (Iwai et al., 2002). C2GnT2, as it has broader acceptor substrate specificity then C2GnT1 or C2GnT3, can act on this disaccharide transferring a GlcNAc to the GalNAc and initiating the Core 4 branch of O-glycans (Figure I.1) (Yeh et al., 1999). In all cases C2GnT2 transfers a GlcNAc from the donor substrate UDP-GlcNAc in a \(\beta\)-1,6-linkage. As with Core 1 and Core 2 O-glycan, the term Core 4 O-glycan is used to refer to O-glycan structures containing both the Core 3 branch and the Core 4 branch, and Core 3 O-glycan refers to structures containing only the Core 3 branch (Figure I.1).

Other common pathways include transferring a sialic acid to the Tn antigen generating the sialyl Tn antigen, and the addition of sialic acid(s) to the T antigen producing the sialyl or disialyl T antigen (Figure I.1) (Marth,
Typically the presence of sialic acid prevents further elongation of that glycan branch, and thus sialic acids are often said to cap glycans. Additionally, the modification of one glycan branch can also influence other O-glycan branches. For example, the addition of a sialic acid to the galactose of the T antigen prevents the initiation of the Core 2 branch (Priatel et al., 2002). However, the presence of the Core 2 branch does not inhibit the elongation of the Core 1 O-glycan branch (Yeh et al., 2001).

With its broader substrate specificity C2GnT2, like Iβ-1,6-N-acetylglucosaminyltransferase (IGnT), is also able to act on linear polylactosamine structures generating branched polylactosamine structures. However, C2GnT2 preferentially transfers GlcNAc to the predistal galactose residue (dl-branching activity) while IGnT preferentially acts on the central galactose (cl-branching activity) (Yeh et al., 1999) (Figure I.2). All of these branches can be further modified by additional glycosyltransferases, generating a wide array of O-glycans. Thus the expression of different O-glycan structures is influenced by many factors including the expression of various competing glycosyltransferases.

The Functions of Mucin-type O-glycans

As Mucin-type O-glycosylation is the best studied of the various types of glycans that can be added to hydroxyl groups, much has been discovered regarding their functions in mammalian physiology. The functions of several
glycosyltransferases and the glycans they generate have been investigated using mouse models deficient for specific glycosyltransferases. It is necessary to study the function of glycosyltransferases and the glycans they generate in the context of intact organisms, as in vitro experiments often fail to reveal functions for glycans (Lowe and Marth, 2003). The failure of cell culture systems to reveal functions for glycosyltransferases and the glycans they generate is likely the result of the extracellular expression of these glycans, which suggests that glycans may often be involved in the homeostasis of multi-cellular systems.

Loss of ppGalNAcT-1 resulted in a range of phenotypes associated with the vascular and immune systems (Tenno et al., 2007). Mice deficient in this glycosyltransferase exhibited a defect in selectin ligand biosynthesis resulting in reduced ability of neutrophils to be recruited to cites of inflammation and reduced cellularity of peripheral lymph nodes. In addition, these mice also had reduced humoral immune responses due to apoptosis of germinal center B-cells. The mechanism leading to abnormal B-cell apoptosis in these mice is currently unresolved. Furthermore, these mice exhibited increased bleeding time, a measure of clotting function, with reduced levels of several coagulation factors including factor V and factor X.

Loss of Core 1 GalT, the glycosyltransferase that initiates Core 1 O-glycans, results in embryonic lethality and hemorrhaging (Xia et al., 2004). The hemorrhaging in these Core 1 GalT-deficient embryos appeared to be due to
altered association of endothelial cells and pericytes. As many cellular adhesion molecules are glycosylated or act as lectins, the defective association of these cell types may be due to altered glycosylation of relevant adhesion molecules.

Core 1 Extension-β1,3-N-acetylglucosaminyltransferase (Core1-β3GnT) has been shown to be required for normal selectin ligand expression, as the elongated Core 1 O-glycans this enzyme generates serve as one scaffold for sLe^x and 6-sulpho-sLe^x, which are the carbohydrates recognized by selectins (Yeh et al., 2001; Marth and Grewal, 2008). C3GnT has been shown to be required to maintain mucosal Muc2 levels, and consequentially to maintain mucosal barrier function and to prevent increased pathology following induction of colitis with dextran sodium sulfate (DSS) (An et al., 2007).

Sialylation of O-glycan structures has also been shown to be required for normal physiology (Lowe and Marth, 2003; Marth and Grewal, 2008). Mice deficient in ST3Gal-I, the sialyltransferase responsible for sialylating the galactose of the Core 1 disaccharide and thus generating the sialyl T-antigen, have reduced levels of CD8^+ T-lymphocytes in circulation due to increased apoptosis of these cells in circulation (Priatel et al., 2000). Apoptosis in these cells has been shown to be similar to the apoptosis normally initiated during the T-cell contraction phase, which follows T-cell expansion resulting from an immune challenge. This suggests that a sialylation state-dependent lectin may be involved in controlling CD8^+ T-cell contraction (Van Dyken et al.,
Additionally, the biosynthesis of selectin ligands is thought to require the action of a $\alpha$-2,3-sialyltransferase (ST3Gal). Six sialyltransferases with this activity have been identified (ST3Gal-I through ST3Gal-VI). Mice singly deficient for ST3Gal-I, ST3Gal-II, ST3Gal-III and ST3Gal-IV have been described, but only ST3Gal-IV has been shown to play a role in selectin ligand biosynthesis (Ellies et al., 2002). However, the defect in selectin ligand biosynthesis in ST3Gal-IV-deficient mice was relatively minor, as these mice did not develop leukocytosis and were able to normally recruit neutrophils to sites of inflammation (Ellies et al., 2002). As ST3Gal-V transfers sialic acids to glycolipids, it is likely that either ST3Gal-VI is required to generate the majority of selectin ligands or that several ST3Gals collaborate in the generation of selectin ligands (Lowe and Marth, 2003).

Fucose residues are also able to decorate O-glycans. Loss of fucosyltransferases has also been shown to result in reduced selectin ligand biosynthesis, and thus defects in leukocyte recruitment and lymphocyte homing (Maly et al., 1996; Homeister et al., 2001).

**C2GnTs and Core 2 O-glycans**

The three glycosyltransferases with C2GnT activity are encoded by Gcnt1 (encoding C2GnT1), Gcnt3 (encoding C2GnT2), and Gcnt4 (encoding C2GnT3). These three genes are highly similar, with the murine genes encoding proteins with greater than 60% similarity, and highly conserved, with
each gene sharing greater than 80% homology with the respective human gene. Additionally, each of these single coding exon genes encodes type-2 membrane proteins with nine conserved cysteine residues (Yen et al., 2003) and one N-glycosylation site (Schwientek et al., 2000), which has been shown to be required for function of C2GnT1 (Toki et al., 1997).

In mammals Core 2 O-glycans are highly expressed (Schachter and Brockhausen, 1989). As generation of a large number of Core 2 O-glycans likely requires a large energy investment by cells, it can be hypothesized that Core 2 O-glycans are physiologically important. C2GnT1-deficient mice have been previously been shown to have an unexpectedly restricted phenotype with the major defect being leukocytosis caused almost entirely by neutrophilia (Ellies et al., 1998). This neutrophilia results from a defect in selectin ligand biosynthesis, leading to a decreased ability of neutrophils to roll on venules and be recruited to some sites of inflammation including the peritoneum (Ellies et al, 1998; Sperandio et al., 2001). C2GnT1-deficient mice also have slightly reduced number of B-cells in their peripheral lymph nodes, as a result of a partial reduction in L-selectin ligand biosynthesis on the lymph node high endothelial venules (HEV) (Hiraoka et al., 2004; Gauguet et al., 2004). C2GnT-1 deficient thymic progenitors have a reduced ability to home to the thymus due to loss of P-selectin ligands on these cells (Rossi et al., 2005). Following the analysis of C2GnT1-deficient mice, C2GnT2 and C2GnT3 were identified (Schwientek, 1999; Yeh et al., 1999; Schwientek et al., 2000).
presence of these three isozymes suggests the possibility of overlapping and distinct functions (Homeister et al., 2001; Akama et al., 2006). However the physiological functions of these isozymes have not been investigated.

It is clear that some Core 2 O-glycans can act as a scaffold for some selectin ligands, but other roles for Core 2 O-glycans are unknown at present (Lowe and Marth, 2003). Studies of Core 2 O-glycan expression changes in normal and disease states have suggested roles for these O-glycans in: T-cell development and activation, MAP kinase pathway signaling and diabetic cardiomyopathy, Wiskott-Aldrich syndrome, Acquired Immune Deficiency Syndrome, and a variety of cancers (Shimodaira et al., 1997; Tsuboi and Fukuda, 1998; Koya et al., 1999; Tsuboi and Fukuda, 2001; Hagisawa et al., 2005). Also, Core 2 O-glycans have been hypothesized to play a role in mucin function (Ellies et al., 1998) because of the high frequency in which mucins are decorated with O-glycans, and the high level of expression of Core 2 and Core 4 O-glycans in mucin-producing tissues (Brockhausen, 2004). These functions include barrier formation, lubrication associated with hydration, and cell-to-cell interactions (Hang and Bertozzi, 2005). Also, underglycosylated mucins have been reported to have a higher rate of endocytosis (Altschuler et al., 2000). Although these studies give us hints as to the possible functions of Core 2 O-glycans, and the C2GnTs that generate them, current understanding fails to reveal why three C2GnTs exist and have been evolutionarily conserved.
In summary

My dissertation research has mostly been focused on investigating the physiological roles of the C2GnTs and the O-glycans they generate. We have accomplished this by determining the phenotypes exhibited in mice singly or multiply deficient in C2GnTs. Individual loss of C2GnT2 or C2GnT3 resulted in vastly different phenotypes, as will be reported in the body of the dissertation. Remarkably, we found that mice deficient for all three C2GnT glycosyltransferases are viable and fertile. Preliminary data from these triply deficient mice suggests that additional phenotypes exist in these mice that do not exist in single C2GnT-deficiency models. This suggests that compensation and collaboration by C2GnTs exists. Additionally, analysis of O-glycan structures in C2GnT deficient mice revealed that C2GnTs play a role in regulating the abundance of unexpected glycan structures, in addition to being required to generate Core 2 and Core 4 O-glycans.
**Figures**

**Figure I.1: Biosynthesis of Mucin-type O-Glycan.** The biosynthesis of common Mucin-type O-glycans is shown. Monosaccharides are depicted as common shapes as indicated.
Figure I.2: I-Branching Activity of C2GnT2 and IGnT. The two types of I-branching activity are depicted. dI-branching activity transfers GlcNAc to the preDistal galactose, and is the preferential I-branching activity of C2GnT2; this is suggested in the figure by listing the C2GnT2 enzyme first. cI-branching activity transfers the GlcNAc to the Central galactose, and is the preferred I-branching activity of IGnT. This preference is depicted in the picture by listing the IGnT glycosyltransferase first for cI-branching activity (Yeh et al., 1999).
References


Yeh, J.C., Ong, E. and Fukuda, M. 1999. Molecular cloning and expression of a novel \( \beta \)-1,6-N-Acetylg glucosaminyltransferase that forms Core 2, Core 4, and I branches. J. Biol. Chem. 274:3215-322

CHAPTER 1:

Biological Functions of Glycosyltransferase Enzymes
Critical in Core O-Glycan Biosynthesis

Abstract

Three conserved glycosyltransferases have been identified that can initiate the formation of the Core 2 branch of mammalian O-glycoproteins. Core 2 O-glycans are abundant in mammalian tissues, but to date few functions for these structures have been defined. To further reveal the biological roles of Core 2 O-glycans, we have generated mice singly and multiply deficient in the three glycosyltransferases that generate Core 2 O-glycans (C2GnT1, C2GnT2 and C2GnT3). A role for C2GnT1 in selectin ligand formation has been described. We report that mice lacking C2GnT2 have decreased mucosal barrier function and increased susceptibility to colitis. C2GnT2 deficiency further results in loss of Core 4 O-glycosylation and reduced abundance of circulating and mucosal immunoglobulins. In contrast, loss of C2GnT3 altered behavior linked to reduced circulating Thyroxine abundance. Remarkably, absence of all three C2GnTs was permissive of viability and fertility. Core 2 O-glycan structures were reduced among tissues from individual C2GnT deficiencies and completely lost in tissues from triply deficient mice. C2GnT deficiency also results in alterations in abundances of other O-glycan
structures. These findings reveal that while the absence of C2GnT function is tolerable, Core 2 O-glycans are important in multiple biological processes.

**Introduction**

Protein O-glycosylation is a posttranslational modification implicated in a wide range of physiological processes including: cell adhesion and trafficking, T-cell apoptosis, cell signaling, endocytosis and pathogen-host interaction (Altschuler et al., 2000; Lowe, 2001; Baum, 2002; Kawakubo et al., 2004; Varki, 2006; Zachara and Hart, 2006; Stanley, 2007). Core-type protein O-glycosylation is initiated in the secretory pathway by the covalent addition of a N-acetylgalactosamine (GalNAc) to the hydroxyl group of serine or threonine residues by one of multiple polypeptide N-acetylgalactosamine transferases (ppGalNAcTs) (Ten Hagen et al., 2003; Tarp and Clausen, 2008). The ppGalNAcTs have differences in substrate specificity, with some ppGalNAcT acting preferentially on specific peptide sequences and others acting only after a GalNAc linkage has been formed nearby (Hassan et al., 2000; Raman et al., 2008). Following protein O-glycosylation by ppGalNAcT activity, other glycosyltransferases act sequentially, and sometimes competitively, to elaborate the repertoire of O-glycan structures (Schachter and Brockhausen, 1989; Perez-Vilar and Hill, 1999; Schachter, 2000; Lowe and Marth, 2003).
The Core 2 β1,6-N-acetylglucosaminylttransferases (C2GnTs) and the Core 2 O-glycans they generate are conserved and widely expressed in mammals. The C2GnTs operate after Core 1 β-1,3-galactosyltransferase (also referred to as T-synthase) adds a galactose in a β1,3-linkage to the GalNAc-Ser/Thr (also referred to as the Tn antigen) generating the initial Core 1 O-glycan disaccharide structure (Ju et al., 2002). Subsequently, one of the three C2GnTs (C2GnT1, C2GnT2 and C2GnT3) can add a N-acetylglucosamine (GlcNAc) in a β1,6-linkage to the GalNAc to initiate what is known as the Core 2 O-glycan branch (Figure 1.1a) (Bierhuizen and Fukuda, 1992; Schwientek et al., 1999; Yeh et al., 1999; Schwientek et al., 2000). In a distinct pathway, Core 3 β-1,3-N-acetylglucosaminylttransferase (C3GnT) can add a GlcNAc to the unmodified GalNAc to generate a Core 3 O-glycan (Iwai et al., 2002). C2GnT2 is then uniquely able to add a GlcNAc in β1,6-linkage to the GalNAc of the Core 3 O-glycan disaccharide to produce a Core 4 O-glycan for subsequent elaboration (Figure 1.1b) (Schwientek et al., 1999; Yeh et al., 1999). In addition, C2GnT2, similarly to the I β-1,6-N-acetylglucosaminylttransferase (IGnT), is able to generate branched polylactosamine structures (I-branches) from otherwise linear polylactosamine glycan chains (Figure 1.1c) (Yeh et al., 1999).

C2GnT1-deficient mice have leukocytosis reflecting neutrophilia (Ellies et al., 1998). This appears to result from an unexpectedly selective defect in selectin ligand biosynthesis among myeloid cells, leading to decreased
recruitment of neutrophils that attenuates inflammation and disease pathogenesis (Ellies et al., 1998; Wang et al., 2008). C2GnT1-deficient mice also exhibit a partial reduction in L-selectin ligand biosynthesis on high endothelial venules (HEV) of lymph nodes, resulting in reduced B-cell homing and colonization of peripheral lymph nodes (Gauguet et al., 2004; Hiraoka et al., 2004). Furthermore, thymic progenitors from C2GnT1 deficient mice have a reduced ability to home to the thymus due to loss of P-selectin ligands on these cells (Rossi et al., 2005). To investigate why multiple glycosyltransferases capable of Core 2 O-glycan formation have been conserved, we have generated and characterized mice singly and multiply deficient in the three C2GnTs.

**Materials and Methods**

**Mice**

Genomic clones isolated from the 129/SvJ mouse strain that included the single coding exon of *Gcnt3*, encoding C2GnT2, and *Gcnt4*, encoding C2GnT3, were used to construct targeting vectors. To generate individual targeting vectors for each gene, the genomic clones and the pflox vector were digested with the appropriate restriction enzymes as indicated (Figure 1.2). The pflox vector was then ligated into each genomic clone generating the targeting vector for each gene. The targeting vectors were then individually electroporated into R1 ES cells (Nagy et al., 1993). Homologous
recombination between the targeting vector and genomic DNA resulted in F[tk-neo] alleles. G418 (to select for neo gene expression) was used to select for cells in which the targeting vectors had integrated. A Cre-recombinase expressing plasmid was electroporated into these cells. Ganciclovir was used to select for colonies in which thymidine kinase (tk) was deleted by homologous recombination. Southern blotting of genomic DNA was done to confirm alleles present. Individual chimeric mice were obtained from C57BL/6NHsd blastocytes injected separately with ES cells containing the alleles in which the single coding exon of interest was flanked by loxP sites. Mice carrying these alleles, Gcnt3F or Gcnt4F, in the germline were crossed with female ZP3-Cre mice to generate separate mice with systematic deletions, Gcnt3Δ or Gcnt4Δ. The mice strains were backcrossed onto the C57BL/6NHsd background.

Mice deficient in multiple C2GnTs were generated by crossing single deficient strains. C2GnT1-deficient mice, which have been previously described (Ellies et al., 1998), were crossed to C2GnT3-deficient mice to generate mice heterozygous for both alleles. These doubly heterozygous mice were bred to each other to generate mice doubly deficient for C2GnT1 and C2GnT3 (T1/T3). T1/T3 mice were bred to C2GnT2-deficient mice to generate mice heterozygous for all three genes encoding C2GnTs. Triply heterozygous mice were bred to each other to generate mice doubly deficient for C2GnT1 and C2GnT2 (T1/T2) and doubly deficient for C2GnT2 and
C2GnT3 (T2/T3) as well as mice deficient for all three C2GnTs (T1/T2/T3).

Some T1/T2/T3 mice were used in additional breedings to generate experimental mice. Mice were used in accordance with Institution Animal Care and Use Committee, University of California, San Diego.

**Quantitative-PCR**

RNA was obtained from wild-type C57BL/6NHsd tissues. Tissues were harvested and stored at -80°C. To isolate the RNA the tissue was placed in TRI-Reagent (Sigma, St. Louis, MO) and homogenized, after homogenization chloroform (Sigma, St. Louis, MO) was added for extraction. RNA was pelleted using isopropanol (Sigma, St. Louis, MO) and cleaned using 70% ethanol. RNA was dissolved in H₂O and treated with Turbo DNA-free (Ambion, Austin, TX) to remove DNA. RNA was run on an agarose-formaldehyde gel to determine quality and stored at -80°C. RNA was quantified using OD₂₆₀ and diluted to 0.5 µg/µl. qPCR was done as previously described with slight modifications (Merzaban et al., 2005). cDNA was generated using 1 µg of RNA and Superscript III First Strand (Invitrogen, Carlsbad, CA). cDNA product was diluted 1/10, and 1 µl of diluted cDNA plus 0.5 µm primers was used with Brilliant SYBR Green (Stratagene, Cedar Creek, TX) for the qPCR reaction. AGGCTCCTCTTCCCTCAAAG was used for the Gcnt4 forward primer and ACATCACCCTCCCTCAAAGTC as the Gcnt4 reverse primer. Results were standardized using β-actin, and data is graphed
relative to testes expression. Microarray analyses by the Consortium for Functional Glycomics suggested testes to be a tissue in which all three C2GnTs may be expressed (Head et al., 2003).

**Selectin ligand expression**

Selectin ligand expression was analyzed as previously described with slight modifications (Ellies et al., 1998; Tenno et al., 2007). Briefly, chimeras consisting of the lectin domains of mouse E- or P-selectin and the Fc portion of human IgG (R&D Systems, Minneapolis, MN) were bound to FITC conjugated anti-human IgG (Fc specific) (Sigma, St. Louis, MO) in binding media, DMEM (Gibco) plus 2% IgG-Free BSA (Jackson ImmunoResearch, West Grove, PA) plus 2 mM Ca or 5 mM EDTA in the dark at 4°C for 30 minutes. White blood cells were washed with binding media and then stained with selectin-Fc chimeras prebound to anti-human Fc-FITC. Selectin chimera binding to white blood cells was determined by flow cytometry using FACSCalibur System (BD Biosciences, San Jose, CA).

To activate T-cells, splenocytes were cultured in RPMI media plus 10% bovine serum in the presence of plate bound anti-CD3 (BD Biosciences, San Jose, CA) and 20 ng/ml IL-2 (R&D Systems, Minneapolis, MN) for 48 hours. At the indicated time points cells were suspended in binding media and expression of activation markers or selectin ligands was determined by flow cytometry.
Hematology

Hematological parameters were determined utilizing a Hemavet 850FS multi-species hematology system (Drew Scientific, Wayne, PA) as previously described (Tenno et al., 2007).

In vivo mucosal permeability assay

Mucosal barrier function was determined as previously described (Furuta et al., 2001). Briefly, dextran-FITC (Sigma, St. Louis, MO) was administered via oral gavage (600 mg/kg) and blood was collected into Microtainer serum separator tubes (BD, Franklin Lakes, NJ) by retro-orbital bleeds at 4 hours. Blood was spun down in a tabletop centrifuge at 16.1 xg for 3 min to separate sera. The amount of FITC in each sample was measured in duplicate via a fluorescent plate reader, using 490 nm and 530 nm for excitation and emission wavelengths, respectively.

DSS-induced colitis

Mice were administered drinking water containing 5% DSS (M.W. 40,000 to 50,000; USB Corporation, Cleveland, OH) ad libitum and then returned to normal drinking water without DSS. In one experiment DSS was administered for 5 days and in another for 6 days. Activity, occult or overt blood in the stool, stool consistency, and weight were determined daily throughout the experiment and used to calculate disease activity index (DAI)
as previously described (Ho et al., 2006). Mice surviving to the end of the experiment were then sacrificed and colons were fixed for histology. The amount of 5% DSS ingested was monitored daily and did not differ between groups. Mice that did not drink enough to surpass a DSS-load of 30mg/day were excluded from the study. Colon sections were stained with hematoxylin and eosin (H&E). The severity of mucosal injury was graded similarly to that described previously by (Okayasu et al., 1990; Murthy et al., 1993). Briefly, H&E stained section were read in a blinded manner to determine length of colon ulceration and crypt damage score. The injury scale was graded from 0 to III, as follows: grade 0: normal; grade I: distortion and/or destruction of the bottom third of glands; grade II: erosions/destruction of all glands or the bottom two thirds of glands and inflammatory infiltrate with preserved surface epithelium; and grade III: loss of entire glands and surface epithelium. Results are reported as total length of complete ulceration (grade III damage) and total crypt damage score as similarly described (Ho et al., 2006).

Mucosal protein assays

A solution containing proteins from the mucosal layer was made as previously described with slight modifications (deVos and Dick, 1991). Briefly, feces were collected in a clean, empty cage for one hour. The feces were weighed, diluted 20 fold in phosphate buffered saline (PBS), and vortexed to make a fecal solution.
Relative mucosa Muc2 levels were determined by ELISA. Briefly, 96-well Maxisorp (Nunc, Rochester, NY) plates were coated overnight at 4°C with fecal solution diluted 10-fold further with PBS. The plates were washed with PBS, blocked with PBS plus 2% IgG-free bovine serum albumin (BSA; Jackson ImmunoResearch, West Grove, PA) for 1 hour at 22°C, and then washed PBS plus 0.05% Tween 20 (PBST; Fisher Scientific, Pittsburg, PA). Antibody to Muc2 antigen H-300 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 0.2 µg/ml in PBS plus 2% BSA was then allowed to bind overnight at 4°C. The plates were then washed, again and coated with the secondary antibody anti-rabbit conjugated to horseradish peroxidase (HRP; Vector Laboratories, Burlingame, CA) diluted 1/1000. Tetramethylbenzidine (TMB; Sigma, St. Louis, MO) was used as a substrate, and the plates were read at 650 nm.

Immunoglobulin analyses

Flat bottom Maxisorp 96-well plates were coated with 5µg/mL of anti-mouse isotype specific antibodies (IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA; BD Biosciences, San Jose, CA) in PBS overnight at 4°C. Plates were blocked with 2% BSA. Sera samples were diluted 1/2,000 (for IgG1, IgG2a and IgA) or 1/10,000 (for IgG3, IgM and IgG2b) and incubated for 2 hours at 22°C. Fecal solutions were diluted 1/100. Plates were washed with PBST. Anti-isotype antibodies conjugated to alkaline phosphatase (AP) were used as secondary
antibodies. Anti-mouse IgG1, IgG2a, IgG2b, and IgG3 conjugated to AP were diluted 1/500 (BD Biosciences, San Jose, CA). Anti-mouse IgM (1/4000) and IgA (1/1000) conjugated to AP were purchased from Sigma (St. Louis, MO). Plates were incubated with secondary for 1 hour at 22°C, washed with PBST and then the AP substrate para-nitrophenyl phosphate (Sigma, St. Louis, MO) was added. Plates were read at 405 nm.

**Behavioral testing**

Behavioral screening included gross physical assessment, analysis of sensorimotor reflexes and the following assays: acoustic startle, prepulse inhibition, hot plate, tail flick, conditioned fear, initiation of movement, rotarod, wire hang, grip strength, cage-top hang test, pole test, and social dominance. These tests were accomplished as previously reported (Arkan et al., 2005; Long et al., 2006). The tube test for social dominance was performed as previously described (Long et al., 2004). Briefly, in this assay mice of opposite genotypes are put into opposite ends of a 30 cm long tube. The mouse that stays in the tube and causes the other to back out is considered to be dominant and is said to have “won” the challenge. If neither mouse backed out of the tube with in 60 sec the challenge was deemed a “tie.” Each mouse was challenged three times against 3 different mice of the opposite genotype.
Determination of thyroid hormone levels

T4 levels were determined by T4 Enzyme Immunoassays (EIA; Monobind Inc., Lake Forest, CA). TSH levels were determined by a two-site chemiluminometric assay at Hillcrest Medical Center of UC San Diego Medical Center.

Thyroid powder supplemented diet

Chow was supplemented with 0.025% porcine thyroid powder (Sigma, St. Louis, MO) as previously described (Marians et al., 2002) with minor modifications. The Purina 5053 base diet supplemented with 0.025% porcine thyroid powder was purchased from Purina Test Diet (Richmond, IN). Thyroid powder supplemented chow was fed to wild-type and C2GnT3-deficient mice for 2 weeks in a conventional vivarium. Mice were analyzed by the tube test for social dominance prior to and following diet supplementation. Additionally, sera were collected at both time points following the tube test for social dominance. Sera were stored at -20°C until used to determine T4 levels.

TRH stimulation assay

Mice were stimulated with TRH as previously described (Yamada et al., 1997) with minor modifications. Briefly, mice were bled at time zero and then immediately administered 5 µg/kg of TRH (Sigma, St. Louis, MO) dissolved in 100 µl of PBS by intraperitoneal injection. Blood was then collected into
serum separator tubes (BD, Franklin Lakes, NJ) at 1 or 2 hours. Blood was spun down in a tabletop centrifuge at 16.1 xg for 3 min to separate sera. Sera were stored in fresh microfuge tubes at -20°C until needed.

**T4 half-life assay**

NHS-LC-biotin (10mg/kg of body weight) (Pierce, Rockland, IL) was injected intravenously into mice. Mice were then bled for time zero as similarly described (Grewal et al., 2008). Blood was collected at additional time points in serum separator tubes. Serum was stored at -20°C until assayed. Glycine was added to sera to quench any additional NHS activity. Relative remaining biotinylated T4 remaining was determined by ELISA. A flat bottom Maxisorp plate was coated overnight at 4°C with anti-T4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/1000. The plates were washed with PBS and then blocked overnight at 4°C with PBS plus 2% BSA. Sera samples were incubated on the blocked plates for 1 hour at 22°C, and the plates were then washed. Streptavidin-HRP (BD Biosciences, San Jose, CA) diluted 1/5000 in PBS plus 2% BSA was allowed to bind for 45 min at 22°C. Plates were washed again, TMB substrate was added, and plates were read at 650 nm.
**Enzyme activity assays**

Tissues from freshly sacrificed mice were immediately flash frozen and stored at -80˚C until used. C2GnT and C4GnT activity from tissue lysates were determined as previously described (Yeh et al., 1999).

**Sample preparation for mass spectrometric analysis**

Murine tissues were prepared for glycomic screening according to methodology described previously (Sutton-Smith et al., 2000). Briefly, murine tissues were homogenized with Tris buffer and sequentially digested with trypsin (Sigma-Aldrich, Dorset, UK) and PNGase F (Roche, West Sussex, UK). N-glycans were separated from peptides/glycopeptides on Sep-Pak cartridges (Waters, Hertfordshire, UK) and O-glycans were released from the latter by reductive elimination using KBH₄ in KOH. To optimize O-glycan extraction from mucinous tissues, especially stomach, the following modifications have been applied on the previous procedure. Stomach samples were homogenized with water and then glycolipids were extracted out with methanol and chloroform. After trypsin digestion, O-glycans were released from the glycopeptide/peptide pool without N-glycan removal. Although trace amounts of N-glycans might be released together with O-glycans, this strategy enables an improved recovery of O-glycans.

After purification on Dowex columns (Sigma-Aldrich, UK), the O-glycan samples were permethylated and then further purified with Sep-Pak cartridges.
O-glycans were eluted in aqueous acetonitrile fractions and then lyophilized. Glycans are normally eluted in the 35% and 50% acetonitrile fractions, therefore only these fractions were subjected to mass spectrometric analysis. All murine tissues were analyzed in duplicates or triplicates.

**Mass spectrometric data acquisition**

Permethylated samples were dissolved in 10 µl of methanol. Then 1 µl of dissolved sample was pre-mixed with 1 µl of matrix (20 mg/ml 2,5-dihydroxybenzoic acid (DHB) in 70% (v/v) aqueous methanol) and spotted onto a target plate. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometric and tandem mass spectrometric (MS/MS) data were acquired on a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems) in the reflectron mode. The potential difference between the source acceleration voltage and the collision cell was set to 1 kV and argon was used as collision gas. The 4700 Calibration Standard kit, calmix (Applied Biosystems), was used as the external calibrant for the MS mode and [Glu1]fibrinopeptide B human (Sigma-Aldrich, UK) was used as an external calibrant for the MS/MS mode.

Comparison of the relative abundance of O-glycans between C2GnT2-deficient, C2GnT3-deficient and T1/T2/T3 mice with wild type mice of various tissues was done by comparing peak heights of molecular ions of similar masses and by comparing the total ion counts of the different classes of
glycans. Data are summarized in Table 1.II. Note that the structural changes reported here are selected based on the potential biological significance for this study. The full O-glycomes of each tissue will be reported elsewhere.

**Statistical analyses**

Significance in the tube test for social dominance was determined by the chi-square test. For all other experiments the student’s t-test was used to determine statistical significance.

**Results**

*Expression of murine Gcnt3 and Gcnt4 RNA*

The three glycosyltransferases that generate Core 2 O-glycans are encoded by separate genes each with a single coding exon in the murine and human genomes. C2GnT1 is encoded by *Gcnt1*, C2GnT2 by *Gcnt3*, and C2GnT3 by *Gcnt4*. The numbering of the genes and the enzymes differ because the genes were named based on β1,6-GlcNAc transferase activity and the enzymes based on Core 2 activity. *Gcnt2* encodes IGnT, which has β-1,6-GlcNAc transferase activity, but is not able to generate Core 2 O-glycans as it is not able to act upon the Core 1 O-glycan as a substrate (Magnet and Fukuda, 1997).

Expression levels of *Gcnt3* in adult wild-type C57Bl/6Nhsd mouse tissues determined by qPCR revealed that *Gcnt3* has high relative expression
in the gastrointestinal tract, similar to the previously determined expression pattern of human GCNT3 (Yeh et al., 1999). Contrary to the expression of human GCNT4 (Schwientek et al., 2000), relatively low levels of the mouse orthologue Gcnt4 were found in the thymus. Our studies revealed highest levels of expression of murine Gcnt4 in the small intestine, liver, and spleen (Figure 1.1d).

**Germline deletion of Gcnt3 and Gcnt4**

Gcnt3 and Gcnt4 were targeted separately for deletion from the mouse germline using embryonic stem (ES) cells. Gcnt3 was targeted using Cre-loxP conditional mutagenesis of the single coding exon of Gcnt3 (Figure 1.2a). ES cells in which the single coding exon of Gcnt3 was flanked by loxP sites were utilized to generate chimeric mice (Figure 1.2b). Gcnt3F mice were bred to mice expressing Cre recombinase, under the control of the Zp3 promoter (Shafi et al., 2000), to generate mice with a systematic deletion of Gcnt3 (Gcnt3Δ). C2GnT2-deficient mice (Gcnt3Δ/Δ) are viable, born at normal Mendelian ratios, and both genders are fertile.

The single coding exon of Gcnt4 was similarly targeted using Cre-loxP mutagenesis (Figure 1.2c). Chimeric mice were generated with ES cells that carried the Gcnt4F allele (Figure 1.2d). Breeding to Zp3-Cre mice was again utilized to produce offspring carrying the Gcnt4Δ allele in the germline (Figure 1.2d). Both genders of mice homozygous for the Gcnt4Δ allele were also born
at expected Mendelian ratios without overt developmental abnormalities and exhibited normal fecundity as adults.

_Hematology and selectin ligand biosynthesis in mice lacking either C2GnT2 or C2GnT3_

As the selectin ligand biosynthesis defect in C2GnT1 deficient mice is incomplete, and as there is precedence for collaboration among glycosyltransferases in the biosynthesis of selectin ligands (Rosen, 2004; Marth and Grewal, 2008), we analyzed mice lacking either C2GnT2 or C2GnT3 for signs of a selectin ligand defect. Hematological analyses revealed that C2GnT3-deficient mice (Gcnt4Δ/Δ), but not C2GnT2-deficient mice, exhibited slight, but significant neutrophilia. No other alterations in hematological profiles were evident in mice singly deficient for C2GnT2 or C2GnT3 (Table 1.I). Additionally, no overt differences were observed in the cellularity of various immune tissues including in the peripheral lymph nodes, or in the expression of various markers on immune cells including B220 (data not shown). To determine if the neutrophilia exhibited by C2GnT3-deficient mice is a result of reduced selectin ligand biosynthesis in the absence of C2GnT3, selectin ligand expression on neutrophils from C2GnT3-deficient mice was analyzed. Using flow cytometric measurements, neutrophils from these mice expressed unaltered P- and E-selectin ligands (Figure 1.3a). C2GnT2-deficient neutrophils also normally expressed ligands for P- and E-
selectins (Figure 1.3b). In addition, the homeostasis of thymocytes within C2GnT3-deficient mice was unaltered (Figure 1.3c), and expression of cell surface markers including 1B11, which is partly dependent upon Core 2 O-glycosylation (Carlow et al., 1999), were expressed normally (Figure 1.3d). Upon stimulation with plate bound anti-CD3 antibody in the presence of IL-2, C2GnT3-deficient T-cells upregulated activation markers including CD69 and 1B11 similarly to activated wild-type T-cells (Figure 1.3e). Selectin ligand expression was also unaltered among these activated T-cells, revealing that C2GnT3 is not required for upregulation of selectin ligands under these conditions.

C2GnT2 deficiency impairs mucosal barrier function and increases pathogenesis of experimental colitis

As Gcnt3 is relatively highly expressed in tissues with high epithelial cell content, we suspected that the gastrointestinal tract might be affected in C2GnT2 deficiency. In fact, C2GnT2-deficient mice, but not C2GnT3-deficient mice, were found to have increased mucosal permeability indicating a defect in the mucosal barrier (Figure 1.4a and data not shown). To test the ability of the gastrointestinal tract of C2GnT2-deficient mice to protect from chemically induced colitis, we used dextran sodium sulfate (DSS) to experimentally induce disease (Ho et al., 2006). There was a trend towards increased weight loss in C2GnT2-deficient mice treated with DSS (Figure 1.4b). Also, during
each separate experiment there was at least one day on which the disease activity score for C2GnT2-deficient mice was significantly worse than for similarly treated wild-type counterparts (Figure 1.4c). At the end of the experiment, H&E stained colon sections were used to determine length and grade of ulceration in these tissues. DSS-treated C2GnT2-deficient mice exhibited significantly more colon damage with increased ulceration and increased damage to the crypts in the colon (Figure 1.4d, 1.4e and 1.4f).

Mucins, glycoproteins that derive a majority of their molecular weight from O-glycans, have been implicated in mucosal barrier function and protection from DSS-induced colitis (Satsangi et al., 1996; van der Sluis et al., 2006). However, mice deficient for C2GnT2 do not appear to have reduced expression of Muc2, the major secretory mucin in the colon (Figure 1.4g).

*Immunoglobulin deficiencies in the absence of C2GnT2*

The serum of C2GnT2-deficient mice had significantly reduced levels of IgG1, IgG2a and IgG2b (Figure 1.5a). There is also a trend towards decreased serum levels of IgG3 and IgA, but these decreases were not statistically significant in the number of animals studied. In contrast to circulating IgA, mucosal IgA abundance was significantly decreased in C2GnT2-deficient mice (Figure 1.5b). Altered mucosal immune homeostasis, including the absence of mucosal immunoglobulins, has previously been associated with susceptibility to DSS-induced colitis (Murthy et al., 2006; Cho,
Absence of C2GnT2 results in a defect in the immune system, characterized by a reduction in immunoglobulin levels that may be associated with increase in disease susceptibility.

*C2GnT3-deficient mice exhibit a behavioral abnormality linked to reduced Thyroxine levels*

Increased fighting was noted between male mice in litters containing at least one C2GnT3-deficient animal, but not among similar C2GnT2 deficient litters. The behavior of male mice lacking C2GnT3 was further analyzed. C2GnT3-deficient male mice exhibited a significant increase in social dominance compared to wild-type male littermates (Figure 1.6a). This assay is often used as a measure of aggression (Crawley et al., 2007).

Hypothyroidism is one cause of aggression in mammals including dogs and horses (Dodman et al., 1995; Aronson et al., 1998; Fatjo et al., 2003; Venero et al., 2005). Further phenotype screening revealed that C2GnT3-deficient mice had a slight but significant decrease of circulating Thyroxine (T4) levels (Figure 1.6b). To determine if the altered behavior observed in C2GnT3 deficiency was a result of insufficient T4 abundance, we fed C2GnT3-deficient mice and wild-type littermates chow supplemented with 0.025% porcine thyroid powder (Marians et al., 2002). Wild-type and C2GnT3-deficient mice fed thyroid-powder supplemented chow achieved similar circulating T4 levels (Figure 1.6c). When these mice were retested in the social dominance assay,
no difference between C2GnT3-deficient and wild-type mice was observed (Figure 1.6d). This finding revealed that the altered behavior observed in C2GnT3-deficient mice is likely due to insufficient T4 levels in circulation.

The thyroid is stimulated to release T4 in response to secretion of Thyroid Stimulating Hormone (TSH) from the pituitary. Thus we investigated the abundance of TSH in circulation in C2GnT3-deficient mice. No difference was observed in circulating TSH abundance (Figure 1.6e). Reduced levels of circulating T4, in the presence of normal levels of TSH, suggests secondary hypothyroidism because thyroid hormone regulation involves a negative feedback loop in which T4 feeds back to the pituitary to reduce TSH secretion (Fliers et al., 2006). Secondary hypothyroidism can be tested by stimulation with thyrotropin-releasing hormone (TRH), the hormone secreted from the hypothalamus that stimulates the pituitary to release TSH (Yamada et al., 1997; Oliverira et al., 2006). No differences were present in the levels of T4 secreted in response to TRH stimulation among wild-type and C2GnT3-deficient mice (Figure 1.6f). This suggests that the slight reduction in T4 levels may not be sufficient to increase TSH levels via the negative feedback loop.

The mild hypothyroidism in C2GnT3-deficient mice may not be secondary hypothyroidism despite normal levels of TSH. To determine if T4 levels are reduced due to decreased half-life in circulation, we compared the in vivo half-life of T4 in wild-type and C2GnT3-deficient mice. To accomplish this we injected N-hydroxysuccinimide (NHS)-biotin and measured the remaining
amount of biotinylated T4 at various time points. No difference in T4 half-life in wild-type and C2GnT3-deficient mice was detected (Figure 1.6g).

*Mice deficient for all three C2GnTs are viable*

The extent to which the three glycosyltransferases with C2GnT activity can biologically compensate for each other is unknown, thus we chose to generate mice deficient for multiple C2GnTs. As all three genes reside on different chromosomes, we accomplished this by crossing the single deficient states to each other. All three possible C2GnT doubly deficient combinations (T1/T2, T1/T3, T2/T3) were generated, and these animals were born without overt abnormalities and appeared normal. From these parental sources, offspring should theoretically include triple-null littermates (T1/T2/T3). Remarkably, mice deficient for all three C2GnTs were born viable and appeared to develop normally to adults. In addition, both male and female T1/T2/T3 mice were fertile (data not shown).

*C2GnT and C4GnT activity in C2GnT deficient mice*

Glycosyltransferase activity assays were used to determine C2GnT and C4GnT enzyme activity levels among tissue lysates from animals bearing single and multiple C2GnT deficiencies. C2GnT2-deficient colon and mesenteric lymph node samples contained significantly reduced C2GnT activity compared to the level of activity in wild-type lysates (Figure 1.7a).
Furthermore, no significant C4GnT activity was detected in any tissue tested from C2GnT2-deficient mice, implying C2GnT2 is the only mammalian enzyme with C4GnT activity (Schwientek et al., 1999; Yeh et al., 1999). In contrast, no significant decrease in C2GnT activity was measured among various tissues analyzed from C2GnT3-deficient mice, despite the fact that the entire coding region of C2GnT3 was similarly deleted (Figure 1.7b). Nevertheless, very little C2GnT activity was detected in tissues from mice deficient for all three C2GnTs (Figure 1.7c).

**O-glycans structures in C2GnT deficiency**

Determining the structural consequences to the glycome resulting from the absence of C2GnT activity is important to ultimately investigate the glycans responsible for the phenotypes observed. O-glycan structures from C2GnT2 and C2GnT3 singly deficient mouse tissues were compared to wild-type samples using mass spectrometric glycomic methodologies (Jang-Lee et al., 2006). A decrease in Core 2 O-glycan structures, along with increases in various Core 1 O-glycan structures, were detected in C2GnT2-deficient stomach, colon, and small intestine samples (Table 1.II). In C2GnT2-deficient stomach and colon tissues, the increased abundance of Core 1 structures included elongated Core 1 branches, several of which bore polylactosamine. In contrast, in the small intestine non-elongated, sialylated Core 1 O-glycan structures are increased. Furthermore, stomach and colon tissues from
C2GnT2-deficient mice exhibited a decrease in I-branching. Surprisingly, elongated O-mannose structures were detected in the stomach of C2GnT2-deficient mice but not wild-type counterparts. O-glycans structures from C2GnT2-deficient kidney samples were unaltered as compared to wild-type kidney structures, consistent with the relative expression of murine C2GnT2.

C2GnT3-deficient tissues contained O-glycan structural changes in the small intestine, stomach and thyroid/trachea samples with decreases in the relative abundance of some Core 2 O-glycan structures relative to Core 1 O-glycan structures in these tissues. No substantial changes in O-glycan structures were noted in the thymus, colon, kidney or liver from C2GnT3-deficient mice (Table 1.II and data not shown). These findings reveal that even in tissues with high relative expression of C2GnT3, loss of this glycosyltransferase does not lead to widespread O-glycan structural changes.

Tissues from T1/T2/T3 mice lacking all three C2GnTs were analyzed and found to lack all detectable Core 2 O-glycan structures among all tissues surveyed by mass spectrometry (MS) including the colon, small intestine, stomach, and kidney (Table 1.II). These results indicate that no other glycosyltransferases appear able to generate Core 2 O-glycans in vivo. Furthermore, small intestine, stomach, and colon samples from T1/T2/T3 mice had a further increase in elongated Core 1 O-glycans compared to tissues lacking a single C2GnT. Surprisingly, no O-glycan structures containing I-branches were detected in stomach and colon samples from these mice.
There was a remarkable increase in elongated O-mannose structures in the stomach of T1/T2/T3 mice, even in comparison with C2GnT2-deficient stomach samples.

**Discussion**

The potential importance of Core 2 O-glycans is demonstrated by the fact that O-glycans of the Core 2 subtype are among the most prevalent of O-glycan structures in mammals (Schachter and Brockhausen, 1989; Brockhausen et al., 2004), and three separate enzymes with C2GnT activity have been conserved in mammalian evolution. Despite this, relatively little has been discovered regarding the potential biological roles of Core 2 O-glycans, particularly those generated by C2GnT2 and C2GnT3. C2GnT1 has been shown to contribute to the biosynthesis of a subset of selectin ligands that support leukocyte trafficking (Ellies et al., 1998), but whether other C2GnTs have similar functions was unknown. In this study we show that the loss of individual C2GnTs led to distinct physiological effects *in vivo* and resulted in unique O-glycan structural changes. Mice deficient for all three C2GnTs and all Core 2 O-glycans are nevertheless viable and fertile.

*Distinct and potential overlapping functions of C2GnTs*

The slight neutrophilia in the absence of an apparent measurable defect in selectin ligand expression in C2GnT3-deficient mice is reminiscent of mice
deficient for α(1,3)fucosyltransferase-IV (FucT-IV). Mice deficient for FucT-IV also exhibited a slight increase (20%) in neutrophil levels in circulation, although the increase was not found to be statistically significant, without a measurable decrease in binding of neutrophils to selectin chimeras (Homeister et al., 2001). Nevertheless, FucT-IV was shown to contribute to selectin ligand function in vivo, as FucT-IV-deficiency resulted in increased leukocyte rolling velocity in microvessels, a process that is dependent on selectin ligands (Weninger et al., 2000). Thus C2GnT3 may contribute to selectin ligand function in vivo, explaining the slight increase in neutrophil levels in circulation in C2GnT3-deficient mice. However, this minor increase in circulating neutrophils is unlikely to alter physiology.

As decreased mucosal barrier function is associated with Inflammatory Bowel Diseases (IBDs) (Stein et al., 1998; Mankertz and Schulzke, 2007), including colitis, it is likely that the increased pathogenesis following induction of disease with DSS and the increased mucosal permeability in C2GnT2-deficient mice is related. Mice deficient for C3GnT, the glycosyltransferase that generates Core 3 O-glycans, were also found to have both decreased mucosal barrier function and increased susceptibility to DSS-induced colitis (An et al., 2007). In C3GnT mice these phenotypes were attributed to reduced Muc2 levels, a mucin known to be necessary for protection from colitis (van der Sluis et al., 2006; An et al., 2007). In contrast, C2GnT2 deficient mice did not appear to have decreased expression of Muc2. However, it is not possible
to determine if reduced mucosal barrier function and increased in susceptibility to induced colitis is partly or completely due to loss of mucin function due to abnormal glycosylation. Susceptibility to IBDs is associated with factors other than altered mucin levels, including changes in commensal and pathogenic organisms as well as altered immune system homeostasis (Xavier and Podolsky, 2007; Peterson et al., 2008). Thus in C2GnT2-deficient mice the impaired humoral immune homeostasis exhibited by reduced serum IgG1, IgG2a, IgG2b, and mucosal IgA levels may contribute to the increased susceptibility to experimental colitis. Thus the loss of these two different, but related glycosyltransferases, increases susceptibility to DSS-induced colitis through different mechanisms. Furthermore, as high relative expression of C2GnT2 is limited to tissues with high epithelial cell content, it is possible that the immune alteration demonstrated by a decrease in Ig subtypes is due to a defect specific to the mucosal immune response.

While the mechanism leading to the reduced T4 abundance in C2GnT3-deficient mice is currently unresolved, remaining possibilities include alterations in the T4 feedback loop or possibly decreases in half-life or response to stimulation below levels of detection in our assays. Alterations in the T4 feedback loop may also explain why TSH levels were not significantly increased in C2GnT3-deficient mice. Furthermore, as C2GnT3-deficient mice exhibited signs of aggression linked to decreased T4 abundance without any
other apparent symptoms of hypothyroidism, increased aggression may be one of the first symptoms to develop in some cases of hypothyroidism.

While each single deficient model exhibited distinct phenotypes, the presence of novel phenotypes in mice deficient for multiple C2GnTs, but not present in any of the singly deficient models, would reveal further collaboration and compensation by C2GnTs. In fact, our preliminary data indicates that T1/T2/T3 mice have elevated levels of the liver enzyme alanine transaminase (ALT), suggesting possible alterations in liver function in the absence of all three C2GnTs (unpublished observation). Thus C2GnTs have distinct and overlapping functions.

*Structural basis of O-glycan biosynthesis as controlled by the three C2GnT glycosyltransferases*

While none of the tissues we tested from C2GnT3-deficient mice had a measurable decrease in C2GnT activity, all three single deficient states have been shown to result in a reduction of in Core 2 O-glycan structures *in vivo* (Ellies et al., 1998). However, the decrease in these structures in C2GnT3 deficient tissue samples was modest, suggesting that C2GnT3 may have a relatively minor contribution to total Core 2 enzyme activity in these tissue types. While no Core 2 O-glycan structures were observed in T1/T2/T3 mice *in vivo*, tissue lysates from these mice had a small amount of C2GnT activity. This suggests that another glycosyltransferase, possibly IGnT, may have
some C2GnT activity in vitro. Alternatively, this residual activity may reflect background noise, indicating that all glycosyltransferases with C2GnT activity have been identified. The large decrease in the abundance of I-branching on O-glycan structures from stomach and colon tissues of mice lacking C2GnT2 suggests that in some tissues C2GnT2, and not IGnT, is the dominant I-branching glycosyltransferase for O-glycans. The further loss of I-branching in stomach and colon samples from triply deficient mice indicates that either C2GnT1 or C2GnT3 has I-branching activity in vivo.

The loss of C2GnTs also alters the expression of other O-glycan structures. For example, some of the elongated Core 1 O-glycan structures generated in the absence of C2GnT activity carry polylactosamines. Altered glycan lectin interactions in these mice may result in altered physiology. This increase in elongated Core 1 O-glycan structures is not likely to be due to reduced competition for acceptor substrates, as it has been previously shown that the initiation of the Core 2 branch does not decrease the efficiency of Core 1 Extension-β1,3-N-acetylglucosaminyltransferase (Core1-β3GlcNAcT) (Yeh et al., 2001). An alternate explanation is that loss of C2GnT activity may lead to an increased abundance of UDP-GlcNAc donor substrate, which is also utilized by Core1-β3GlcNAcT, resulting in increased Core 1 elongation. Reduced competition for UDP-GlcNAc may also explain the surprising induction of elongated O-mannose structures in the T1/T2/T3 stomach tissue, as UDP-GlcNAc is also the donor substrate for protein-O-mannose-β1,2-N-
acetylglucosaminyltransferase 1 (POMGnT1) (Yoshida et al., 2001; Zhang et al., 2002; Lengeler et al., 2008). Mutations leading to decreased POMGnT1 activity result in a rare limb-girdle muscular dystrophy (Clement et al., 2008; Guglieri et al., 2008). Muscular dystrophies that result from reduced glycosylation of \( \alpha \)-dystroglycan (\( \alpha \)DG) are termed dystroglycanopathies (Grewal and Hewitt, 2003; Martin, 2005). Increasing glycosylation of \( \alpha \)DG has shown potential as a therapy for dystroglycanopathies (Brockington and Muntoni, 2005; Martin, 2007). Our research suggests that it may be possible to increase glycosylation of \( \alpha \)DG in pathologies caused by decreased POMGnT1 activity if C2GnT activity could be inhibited, or UDP-GlcNAc concentration could be increased in relevant cell types.

Our results suggest that each glycosyltransferase with Core 2 activity has a distinct function, and may explain why three separate enzymes with this activity have been conserved through evolution. However, it is possible that additional phenotypes not revealed by our assays may exist in mice deficient for C2GnTs. Additional studies are necessary in order to determine what other phenotypes may exist in these model(s). Perhaps loss of C2GnT2 may alter the ability of commensal or pathogenic bacteria to colonize the host, as it has been shown that *Helicobacter pylori* and *Clostridium perfringens* can interact with Core 2 O-glycan structures (Kawakubo et al., 2004; Ashida et al., 2008). Further studies may reveal a role for C2GnT3 in T-cell function, as previous studies have shown that C2GnT3 is expressed in activated T-cells.
(Merzaban et al., 2005). In fact, preliminary data suggests that C2GnT3 is required for a normal T-cell response following specific intestinal challenge (H. Ziltener, personal communication). Yet, the increase in elongated Core 1 O-glycan structures may serve to compensate to some extent for loss of C2GnTs. Further biological interrogation of mice deficient for C2GnT activity and Core1-β3GlcNAcT would be needed in an effort to address the possibility of compensation by elongated Core 1 O-glycan structures. Nevertheless, these C2GnT deficient mice models have contributed significantly to the understanding of Core 2 O-glycan biology and the biosynthesis of O-glycans.
Tables

Table 1.I: Hematology in the absence of C2GnT2 or C2GnT3. Relative hematological findings for mice singly deficient for C2GnT2 or C2GnT3 are shown. Data is depicted as percent of wild-type results from respective littermate controls. *, p<0.05. Although statistically significant the slight increase in circulating neutrophils in mice lacking C2GnT3 is unlikely to alter physiology.

<table>
<thead>
<tr>
<th></th>
<th>C2GnT2Δ/Δ (percent of wild-type)</th>
<th>C2GnT3Δ/Δ (percent of wild-type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Blood Cells</td>
<td>98.61 ± 3.191</td>
<td>110.9 ± 8.245</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>92.56 ± 8.038</td>
<td>131.8 ± 12.58*</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>99.76 ± 3.182</td>
<td>102.8 ± 7.455</td>
</tr>
<tr>
<td>Monocytes</td>
<td>91.25 ± 5.524</td>
<td>121.4 ± 15.23</td>
</tr>
</tbody>
</table>
Table 1.II: O-glycan structures in the presence and absence of C2GnTs.
The structural changes described here are based on MALDI-TOF/TOF MS and MS/MS data. WT, wild-type mice; C2GnT2Δ/Δ, C2GnT2-deficient mice; C2GnT3Δ/Δ, C2GnT3-deficient mice; T1/T2/T3, C2GnT triple-deficient mice; NA, not analyzed; ND, none detected; =, no significant changes; ser, serine; thr, threonine; upward arrows represent increase; downward arrows represent decrease; the number of arrows is indicative of the magnitude of change with 3 arrows being the greatest (>75%) and 1 arrow the smallest (<25%) percent of change from wild type. Linkages are assigned according to the biosynthetic pathways known. Yellow squares = N-acetylgalactosamine (GalNAc); Blue squares = N-acetylglucosamine (GlcNAc); Half blue and half yellow squares = GalNAc or GlcNAc; yellow circles = galactose; green circles = mannose; purple diamonds = N-acetylneuraminic acid; white diamonds = N-glycolylneuraminic acid; red triangles = fucose.

<table>
<thead>
<tr>
<th>Structural characteristics</th>
<th>Examples of structure</th>
<th>Changes relative to WT</th>
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<tbody>
<tr>
<td></td>
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<td>C2GnT2Δ/Δ</td>
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<tr>
<td>Stomach</td>
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<tr>
<td>Core 2</td>
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<td>Elongated Core 1</td>
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<td>I- Branches</td>
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<tr>
<td>Elongated O-mannose</td>
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<td>Colon</td>
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<tr>
<td>Core 2</td>
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<tr>
<td>Elongated Core 1</td>
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<tr>
<td>I- Branches</td>
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<tr>
<td>Small Intestine</td>
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<tr>
<td>Core 2</td>
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<tr>
<td>Core 1 &amp; Elongated Core 1</td>
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<tr>
<td>Kidneys</td>
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<td>Core 2</td>
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<td>Core 1 &amp; Elongated Core 1</td>
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<tr>
<td>Thyroid / Trachea</td>
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<tr>
<td>Core 2</td>
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<tr>
<td>Core 1 &amp; Elongated Core 1</td>
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<td>NA</td>
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<tr>
<td>Sph⁪</td>
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<td>NA</td>
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<tr>
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<tr>
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<tr>
<td>Core 1 &amp; Elongated Core 1</td>
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**Figures**

(a) Biantennary Core 2 O-glycans are generated when any of the three C2GnTs acts on the Core 1 O-glycan disaccharide. (b) C2GnT2 can generate Core 4 O-glycans from Core 3 O-glycans by adding a GlcNAc to the initiating GalNAc. (c) C2GnT2, in addition to IGnT, also has the ability to generate branched polylactosamine repeats from linear polylactosamine repeats. The figure depicts distal I-branching as the GlcNAc is transferred to the predistal galactose, the preferential I-branching activity of C2GnT2. (d) Relative RNA expression of murine Gcnt3 (left panel) and Gcnt4 (right panel), which code for C2GnT2 and C2GnT3, respectively, as determined by qPCR. All values are means ± S.E.M.

**Figure 1.1:** Activity and expression of C2GnTs. (a-c) Monosaccharides are depicted as geometric shapes with GalNAc as a yellow square, galactose as a yellow circle, and GlcNAc as a blue square. In addition, the vertical arrows indicate that each branch can be further elaborated by additional saccharide linkages. (a) Biantennary Core 2 O-glycans are generated when any of the three C2GnTs acts on the Core 1 O-glycan disaccharide. (b) C2GnT2 can generate Core 4 O-glycans from Core 3 O-glycans by adding a GlcNAc to the initiating GalNAc. (c) C2GnT2, in addition to IGnT, also has the ability to generate branched polylactosamine repeats from linear polylactosamine repeats. The figure depicts distal I-branching as the GlcNAc is transferred to the predistal galactose, the preferential I-branching activity of C2GnT2. (d) Relative RNA expression of murine Gcnt3 (left panel) and Gcnt4 (right panel), which code for C2GnT2 and C2GnT3, respectively, as determined by qPCR. All values are means ± S.E.M.
Figure 1.2: Generation of mice singly deficient for C2GnT2 or C2GnT3.
(a) Gcnt3 genomic clone from 129/SvJ mouse strain was used to generate a targeting construct using the pflox vector as indicated. B: Bgl II, Ba: Bam HI, E: Eco RI, S: Spe I, X: Xba I, Xh: Xho I. (b) Southern blotting of genomic DNA confirms the Gcnt3 allele structure present in ES cells using the genomic probe (top). Southern blotting with a loxP probe detects the location and number of loxP sites (bottom). (c) The targeting of the single coding exon of Gcnt4 using the pflox vector is depicted. A: Age I, B: Bam HI, E: Eco RV, S: Sac I, Sa: Sal I, St: Stu I, X: Xho I. (d) Southern blots of genomic DNA with the genomic probe (top) or loxP probe (bottom) indicate the structure of Gcnt4 alleles present.
Figure 1.3a-c: Selectin ligand expression on neutrophils and T-cells from C2GnT-deficient mice. (a and b) Histograms depict the expression of ligands for P- and E-selectins on neutrophils from mice singly deficient for (a) C2GnT3 or (b) C2GnT2. Addition of EDTA controls for binding of C-type lectins. (c) Number of thymocytes of each cell type in wild-type and C2GnT3-deficient mice is graphed (DN: CD4⁻, CD8⁻ cells; DP: CD4⁺, CD8⁺ cells; SP: CD4⁺ or CD8⁺ cells).
Figure 1.3d-e: Selectin ligand expression on neutrophils and T-cells from C2GnT-deficient mice. (d) Expression of the 1B11 antigen on thymocyte sub-populations is shown. (e) Histograms indicate the level of expression of activation markers and selectin ligands on activated wild-type and C2GnT-deficient T-cells. All values are means ± S.E.M.
Figure 1.4: Barrier function and colitis in C2GnT2-deficient mice. (a) The amount of dextran-FITC in sera four hours after administration by gavage is graphed. Data shown is from a minimum of 6 mice per a genotype. (b) Graph shows the average percent weight change as compared to t=0 of wild-type (closed squares) and C2GnT2-deficient (open squares) mice during and following treatment with 5% DSS until onset of mortality. The horizontal line represents the time of the DSS treatment. DSS was given to 6 mice of each genotype. (c) DAI of wild-type and C2GnT2-deficient mice treated with 5% DSS is graphed. (d) The length of grade III damage (total ulceration with loss of glands and surface epithelium) in colon sections from of DSS-treated wild-type and C2GnT2-deficient mice is shown. (e) The average crypt damage score is graphed. (f) Representative H&E stained colon sections from DSS-treated wild-type mice (illustrating grade II and grade I crypt damage) and C2GnT2-deficient mice (illustrating grade III crypt damage or total ulceration). (g) Relative mucosal Muc2 levels from untreated wild-type and C2GnT2-deficient mice are graphed. All values are means ± S.E.M. S.E.M. is represented by capped and uncapped vertical lines. (p<0.05)
Figure 1.5: Circulating and mucosal immunoglobulins in wild-type and C2GnT2-deficient mice. (a) Circulating levels of immunoglobulins of the IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA isotypes in wild-type and C2GnT2-deficient mice is graphed. Data shown is pooled from two separate experiments, each of at least 5 mice of each genotype per an experiment. (b) Mean mucosal IgA levels in wild-type and C2GnT2-deficient mice fecal samples are graphed. All values are means ± S.E.M. (*p<0.05)
Figure 1.6: Behavior and thyroid function in C2GnT3-deficient mice. (a) Results from the tube test for social dominance are graphed. The results shown are representative of three separate experiments. (b) Mean circulating T4 levels in wild-type and C2GnT3-deficient mice is graphed. (c) Mean circulating T4 levels in wild-type and C2GnT3-deficient mice following diet supplementation with 0.025% thyroid powder for 2 weeks is shown. (d) Outcome of social dominance assay performed with wild-type and C2GnT3-deficient mice treated with 0.025% thyroid powder supplemented chow is shown. (e) Mean circulating levels of the TSH in wild-type and C2GnT3-deficient mice is graphed. (f) Average amount by which TRH stimulation increased T4 in circulation after 1 and 2 hours in wild-type and C2GnT3-deficient mice is shown. (g) The mean relative amount of biotinylated T4 remaining at each time point is graphed. All capped and uncapped error bars represent S.E.M. (*p<0.05; **p<0.01)
Figure 1.7: Enzyme activity in tissue lysates from mice deficient for C2GnTs. (a) Relative C2GnT and C4GnT activity in tissue lysates from C2GnT2-deficient mice is graphed. (b and c) C2GnT activity in tissue lysates from (b) C2GnT3-deficient and (c) triply deficient mice relative to activity in wild-type control tissues is shown. Values represent means ± S.E.M. (*p<0.05, **p<0.001)
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References


CHAPTER 2:

Loss of the Glycosyltransferase C2GnT2 Impairs the Immune Response to a Mucosal but Not a Parenteral Antigen

Abstract

Mice deficient for Core 2 β1,6-N-acetylglicosaminyltransferase2 (C2GnT2) have altered homeostasis of circulating and mucosal immunoglobulins. It now appears that the reduced static abundance of immunoglobulins in C2GnT2-deficient mice is a result of a specific inability to generate an antigen-specific humoral response to mucosal but not parenteral antigens. Furthermore, glycoproteins in the mucosa of C2GnT2-deficient mice have altered expression of ligands for Galectin-1, a lectin that has previously been shown to modulate immune responses. Further study of the systemic and mucosal immune responses in C2GnT2-deficient mice may lead to a better understanding of the regulation of the systemic response to mucosal antigens and pathogens.

Introduction

Mucosal surfaces offer unique challenges for an organism as they are in direct contact with the environment. These surfaces are the largest areas in humans in contact with the environment, and thus pathogens have regular
access to mucosal surfaces (Bajaj-Elliott and Sanderson, 2004). In fact, 90% of all infectious agents enter through mucosal surfaces (Bouvet and Fischetti, 1999). However, the host must also remain tolerant of dietary antigens and the large number of commensal organisms colonizing the mucosa, especially in the colon. For this reason a complex mucosal immune system, which includes epithelial cells, a mucus layer of protection, and specialized mucosal innate and adaptive immune cells and molecules, has been developed to protect mucosal surfaces. The complex mucosal immune system includes the largest arm of the adaptive immune system; this immune system is larger in area, cell number, and complexity than the systemic adaptive immune system (Brandtzaeg et al., 1999; McGhee and Kitono, 1999). Dysregulation of this system due to environmental and/or genetic alterations can have disastrous consequences including development of Inflammatory Bowel Diseases (IBDs), which include ulcerative colitis (UC) and Crohn’s disease, Celiac Disease, and food allergies, or on the other hand increased susceptibility to infection caused by immunodeficiency.

A common modification to proteins of both the immune system and mucosa is mucin-type O-glycosylation. Mucin-type O-glycosylation is initiated in the Golgi by the covalent addition of a N-acetylgalactosamine (GalNAc) from an UDP-GalNAc to the hydroxyl group of serine or threonine residues by one of many polypeptide N-acetyl-α-galactosaminyltransferases (ppGalNAcTs). Various competing glycosyltransferases can then act
sequentially to elongate, branch, or decorate the glycan, resulting in the
generation of many different O-glycan structures (Lowe and Marth, 2003). In
mammals, a commonly occurring subtype of O-glycosylation is the Core 2 O-
glycan subtype. Core 2 O-glycans are generated after Core 1 β-1,3-
galactosyltransferase (Core 1 GalT) adds a galactose to the initiating GalNAC
generating the Core 1 structure (Marth, 1999). One of three Core 2 β1,6-N-
acetylgalactosaminyltransferases (C2GnT1, C2GnT2 and C2GnT3), can then
act on the unmodified Core 1 structure to add a \(N\)-acetylglucosamine (GlcNAc)
via a \(\beta\)1,6-linkage to the GalNAC to initiate what is known as the Core 2 branch
(Figure 1.1a) (Schwientek et al., 2000). In an alternative pathway, C3GnT can
add a GlcNAc to the unmodified initiating GalNAC to generate a Core 3 O-
glycan. C2GnT2 can then act on the Core 3 O-glycan to add a GlcNAc via the
same \(\beta\)1,6-linkage to the GalNAC to generate a Core 4 O-glycan (Figure 1.1b)
(Schwientek et al., 1999; Yeh et al., 1999). Core 2 and Core 4 O-glycans can
then be further modified by additional glycosyltransferases. Additionally,
C2GnT2, like I \(\beta\)-1,6-N-acetylgalactosaminyltransferase (IGnT), is able to
generate branched polylactosamine structures from linear polylactosamine
structures, thus C2GnTs, and especially C2GnT2, are key branching enzymes
in controlling O-glycan diversity (Figure 1.1c) (Yeh et al., 1999). Furthermore
Core 2, Core 4 and I-branching are thought to be relatively common
modifications in mucin-producing tissues, and C2GnT2 has been reported to
be primarily expressed in the mucin-producing tissues (Magnet and Fukuda,
Mucin-type O-glycosylation has been shown to be an important factor in immune and mucosa homeostasis. In the immune system O-glycosylation has been shown to regulate immune cell development, trafficking, survival, and activation (Reviewed in van Kooyk and Rabinovich, 2008). Glycosylation is regulated in many leukocytes, in fact Core 2 O-glycan expression has been reported to be regulated during T-cell activation and maturation of dendritic cells (DCs) and by various cytokines. This suggests that Core 2 O-glycosylation of proteins from these cell types may be important for effector functions (Carlow et al., 2001 and Bax et al., 2007), possibly by altering the expression of Galectin ligands on these cell types.

Galectins have been shown to have multiple roles in modulating immune responses. For example, galectin-1 has been shown to increase immunoglobulin production, to play a role in CD8αα T-lymphocyte development, and to alter cytokine secretion from T-cells (Tsai et al., 2008; Liu et al., 2008 and Stowell et al., 2008). Galectin-3 has been implicated in the survival of activated B-lymphocytes and the amplification of T-cell responses by DCs (Acosta-Rodriguez et al., 2004; Breuilh et al., 2007).

O-glycans have been hypothesized to play a role in mucin function because of the high frequency in which mucins, heavily O-glycosylated glycoprotein, are decorated with O-glycans. These functions include barrier
formation, lubrication associated with hydration, and cell-to-cell interactions (Hang and Bertozzi, 2005). Additionally, altered glycosylation has been shown to increase the rate of endocytosis of Muc1 (Altschuler et al., 2000). Furthermore, altered glycosylation is an early event in the development of ulcerative colitis (Corfield et al., 2001). Additionally, loss of the glycosyltransferase Core 3 β-1,3-N-acetylglycosaminyltransferase (C3GnT) has been shown to result in reduced expression of Mucin 2 (Muc2), the major mucin of the colon, which is likely the cause of reduced barrier function and increased susceptibility to experimental colitis in these mice (An et al., 2007).

C2GnT2 have reduced mucosal barrier function and increased susceptibility to experimentally induced colitis (Chapter 1). C2GnT2-deficient mice also have significantly reduced circulating IgG1, IgG2a and IgG2b and mucosal IgA levels (Chapter 1). Thus, to further understand why loss of this glycosyltransferase, which has expression and activity essentially limited to mucin-producing tissues and mesenteric lymph nodes, results in reduced circulating and mucosal immunoglobulin levels (Yeh et al., 1999; Chapter 1), we have further investigated humoral immunity in C2GnT2-deficient mice. Our results show that an effective systemic humoral response to a mucosal antigen but not a parenteral antigen requires C2GnT2. To our knowledge this is the first model found to have a specific reduction in systemic antigen-specific IgGs in response to keyhole limpet hemocyanin (KLH) administered
via the oral route but no difference in the humoral response following intraperitoneal (i.p.) immunization.

**Results**

*C2GnT2-deficient mice generate a normal humoral response to a parenteral antigen*

To further understand the altered immunoglobulin homeostasis in C2GnT2-deficient mice, we immunized C2GnT2-deficient mice with KLH conjugated to 2,4-dinitrophenyl hapten (DNP) emulsified in Complete Freund’s Adjuvant (CFA) administered via i.p. injection, followed by a booster immunization with KLH-DNP plus Incomplete Freund’s Adjuvant (IFA) and determined the antigen-specific response (Tenno et al., 2007). The antigen-specific humoral response to an antigen administered via i.p. immunization in C2GnT2-deficient mice was not different than the response in wild-type mice (Figure 2.1).

*Reduced humoral response to a mucosal antigen in C2GnT2-deficient mice*

We further reasoned that as the expression of C2GnT2 is essentially limited to the mucosa (Yeh et al., 1999, Chapter 1), the altered immunoglobulin homeostasis might be due to an abnormality in the humoral response to mucosal antigens. To test this hypothesis, we immunized C2GnT2-deficient mice and wild-type controls with KLH-DNP plus cholera
toxin (CT), as an adjuvant, via the mucosal route (oral gavage) (Kjerrulf et al., 1997). We observed that C2GnT2-deficient mice had a reduction in antigen-specific IgG\textsubscript{2a}, IgG\textsubscript{2b} and IgG\textsubscript{3} in sera following mucosal immunization with the antigen KLH-DNP (Figure 2.2a). Furthermore there was a trend towards decreased antigen-specific mucosal IgA in C2GnT2-deficient mice following mucosal immunization (Figure 2.2b). Thus, C2GnT2-deficient mice appear to have decreased systemic and possibly mucosal humoral response to KLH administered perorally but not to the same antigen injected intraperitoneally.

\textit{C2GnT2-deficient mice have elevated expression of Galectin-1 ligands in the Mucosa}

Mucin-producing tissues from C2GnT2-deficient mice have an unexpected increase in elongated Core 1 O-glycans, and some of these new structures included polylactosamines (Chapter 1). As polylactosamine can sometimes serve as ligand for endogenous lectins, we further analyzed the O-glycan structures present in colon, small intestine, and stomach of C2GnT2-deficient mice in comparison to potential animal lectin ligands as determined by the Consortium for Function Glycomics (Smith, 2009). This analysis revealed that the mucosa of C2GnT2-deficient mice have increased expression of several glycan structures that have the ability to bind to mammalian lectins and glycan binding proteins. Several of the lectins and glycan binding proteins for which C2GnT2-deficient tissues have altered
expression of potential ligands have been shown to modulate immune responses. These lectins and glycan binding proteins include various galectins, \(\gamma\delta\)T-cell receptors, and several C-type lectins. Glycan structures with altered expression in the mucosa of C2GnT2-deficient mice identified by this approach and the lectins for which they are potential ligands are shown in Table 2.1. Additionally, using this approach we also identified various bacterial lectins and toxins for which the mucosa of C2GnT2-deficient mice show altered expression of potential ligands.

We then chose to further investigate if the altered expression of these potential ligands resulted in altered binding of glycoproteins from C2GnT2-deficient mice to these lectins. As galectins often bind similar ligands and as Galectin-1 has been previously shown to be able to modulate a wide range of immune functions (Camby et al., 2006), Galectin-1 was used as a model lectin for this study. Using an ELISA-like approach, we determined the binding of recombinant Galectin-1 to fecal proteins from wild-type and C2GnT2-deficient mice. We found that fecal proteins from C2GnT2-deficient mice had a trend towards increased expression of Galectin-1 ligands (p=0.0554; Figure 2.3). Mucosal proteins from C2GnT2-deficient mice are also likely to have increased expression of ligands for other lectins that modulate the immune response.
Preliminary analysis of cell type frequencies in Gut-associated lymphoid organs from C2GnT2-deficient mice.

To begin to understand why C2GnT2-deficient mice have a reduced antigen-specific humoral response following mucosal immunization but not following i.p. immunization with the same antigen, we investigated the cell types present in immune organs in C2GnT2-deficient mice and wild-type littermate controls. There were no apparent differences in the frequencies of various cell types in the spleen, thymus or peripheral lymph nodes of C2GntT2-deficient mice in comparison to wild-type mice (data not shown). Cellularity and absolute levels of various cell types also appeared normal in these organs in C2GnT2-deficient mice.

As the expression and activity of C2GnT2 appears to be essentially limited to mucin-producing tissues and the mesenteric lymph node (Yeh et al., 1999; Chapter 1), we investigated the frequency of immune cell types in the gut-associated lymphoid tissues (GALT). Preliminary studies did not find any cell types in the mesenteric lymph node with altered frequency or absolute cell number (Figure 2.4a). However, preliminary results show a decrease in frequency of γδT-cells in the C2GnT2-deficient Peyer’s patches (p=0.00559, n=3) (Figure 2.4b). γδT-cells accounted for 20.1% of cells present in single cell suspension of Peyer’s patches from wild-type mice, but only 14.5% of cells from C2GnT2-deficient Peyer’s patches. However, this decrease in frequency did not result in a significant decrease in absolute abundance of γδT-cells in
Peyer's patches from C2GnT2-deficient mice in these mice. No other significant differences in the frequencies or absolute abundances of various immune cell types were noted in this preliminary study. Additional analysis of immune type frequency and cellularity is justified based on this preliminary study.

In addition, as certain cell surface markers (CD45R-generating the B220 antigen, and CD43 – generating the 1B11 antigen) are known to be modified by Core 2 O-glycans (Ellies et al., 1998; Carlow et al., 1999), and as the interaction between other cell surface markers (CD40 and CD40L) can be modulated by C2GnT1 (Tsuboi and Fukuda, 2001), we determined the cell surface expression of these molecules on immune cells from C2GnT2-deficient GALT. No difference in the expression of B220 or CD40 on B-cells, or 1B11 or CD40L on T-cells was found in the absence of C2GnT2 (data not shown).

Discussion

The mucosal immune system is charged with responding to unique challenges present at mucosal surfaces. It must be ready to respond to the onslaught of microbes and pathogens that frequently enter through mucosal surfaces, and concurrently tolerate commensal organisms and food antigens that are also found at these sites. To accomplish this, the mucosal immune system has developed unique characteristics to identify and cope with these
challenges. While there has been an explosion of knowledge in the field of mucosal biology as of late, the mechanistic basis by which systemic responses are generated to mucosal antigens is still relatively poorly understood. Systemic immune responses are essential for the clearance of some mucosal pathogens and are necessary for the full effectiveness of mucosal vaccines (Bry and Brenner, 2004; Maaser et al., 2004; Mestecky et al., 2008). Thus, understanding the systemic immune responses to mucosal antigens is an important area of research.

Mice deficient for C2GnT2 appear to have a specific defect in the ability to generate normal humoral responses, including systemic humoral responses, to antigens delivered perorally but not intraperitoneally. It is important to realize that the mucosal and i.p. immunizations utilized different adjuvant, CT and CFA, respectively. Thus, it would be necessary to compare the ability of CT to serve as an adjuvant for i.p. administered antigens in C2GnT2-deficient mice and wild-type littermate controls to determine whether the difference in the inability of C2GnT2-deficient mice to generate a normal humoral response following mucosal immunization with KLH-DNP plus CT was due to route of administration or the adjuvant. Furthermore, it is not known if C2GnT2-deficient mice are able to generate a normal antigen-specific humoral response following i.p. immunization with other T-dependent antigens, such as OVA, or other types of antigens, such as the T-independent type 2 antigen ficoll. It is also unknown if C2GnT2-deficient mice are able to
generate normal antigen-specific humoral responses following immunization via other parenteral routes of administration including subcutaneous, intramuscular or intravenous administration. Thus more information regarding the humoral response to various antigen types and routes of entry is necessary in order to determine if the reduced antigen-specific humoral response in C2GnT2-deficient mice is truly specific for only mucosal antigen. However, considering the restricted expression of C2GnT2 (Yeh et al., 1999; Chapter 1), it seems likely that this glycosyltransferase is responsible for mucosal specific functions.

Perorally immunized C2GnT2-deficient mice had a reduced ability to generate antigen-specific IgG2a, IgG2b, and IgG3, the same immunoglobulin isotypes that T-bet deficient B-cells had reduced ability to switch to (Peng et al., 2002). Thus one possible explanation for the reduced ability of C2GnT2-deficient mice to generate antigen-specific IgG2a, IgG2b, and IgG3 following mucosal immunization is that T-bet is not efficiently induced following mucosal immunization of C2GnT2-deficient mice. Reduced T-bet induction could occur if C2GnT2-deficient mice had a reduced IFNγ response following mucosal immunizations, or if C2GnT2-deficient mucosal B-cells had reduced ability to bind IFNγ. This might occur if glycosylation of the IFNγ receptor was altered on C2GnT2-deficient mucosal B-cells.

However, it seems unlikely that the reduced humoral response to mucosal antigens in C2GnT2-deficient mice is B-cell intrinsic, as C2GnT2-
deficient B-cells, including mucosal B-cells, have normal expression of B220, a Core 2 O-glycan-dependent antigen. This suggests that C2GnT2 is not required for proper Core 2 O-glycosylation in these cells. While no difference in the expression of the Core 2 O-glycan-dependent antigen 1B11 was seen on C2GnT2-deficient mucosal T-cells, it is not known if these cells normally upregulate 1B11 expression following activation.

Unimmunized C2GnT2-deficient mice had reduced circulating abundance of IgG₁ (Chapter 1), but normally produced antigen-specific IgG₁ to a T-dependent mucosal antigen. Thus it is possible that C2GnT2-deficient mice will have a reduced antigen-specific humoral response following immunization with other types of antigens. Furthermore, it might be expected that the antigen-specific IgG₁ response may be reduced for these other types of antigens.

The altered expression of Galectin-1 ligands in the mucosa of C2GnT2-deficient mice is of interest as galectins, including Galectin-1, have been shown to be able to modulate immune responses in a variety of ways (Salatino et al., 2008). In fact, Galectin-1 has been shown to induce apoptosis of activated T-cells (Campy et al., 2006). Thus, increased expression of Galectin-1 ligands may lead to increased T-cell apoptosis. The only significant difference in absolute cell number or cell frequency in the GALT of C2GnT2-deficient mice was a decrease in the frequency of γδT-cells in Peyer's patches. However, this preliminary study was performed on a small number of animals,
and it remains possible that the frequency of additional subsets of T-cells will prove to be altered when more animals are analyzed, or when intraepithelial and lamina propria T-cells are analyzed. However, as Galectin-1 specifically induces apoptosis in activated T-cells it may prove necessary to determine activated T-cells frequencies in perorally immunized mice or by using a model mucosal infection. Galectin-1 has also been shown to modulate T-cell responses through the suppression of IFNγ, IL-2, and TNFα (Camby et al., 2006). The modulation of these specific cytokines may partially explain the reduced humoral response in C2GnT2-deficient mice.

In contrast with the reported increased pathogenesis of colitis in C2GnT2-deficient mice following induction of disease with dextran sodium sulfate (Chapter 1), Galectin-1 has been shown to protect from intestinal inflammation following disease induction with 2,4,6-trinitrobenzene sulfonic acid (TNBS) model (Hokama et al., 2008). It is possible that constitutive over-expression of Galectin-1 or Galectin-1 ligands results in an immunodeficiency state that would then be more susceptible to experimental colitis, and that exogenous administration of Galectin-1 following chemical induction of colitis results in disease suppression due to the later and shorter exposure to the lectin. However, other galectins, including Galectin-4, intensify pathogenesis of colitis due to stimulation of CD4+ T-cells (Hokama et al., 2008). Thus, it is possible that the cumulative effect of the altered expression of ligands for various galectins in the mucosa of C2GnT2-deficient mice is a reduction in
humoral responses and increased susceptibility to experimentally induced colitis.

It is of additional interest that potential carbohydrate ligands for \(\gamma\delta T\)-cells were increased in mucosa of C2GnT2-deficient mice. One possibility is that one or more of these potential carbohydrate ligands may be inhibitory for these cells. If this were the case, the increased expression of these potential ligands might explain the decreased frequency of \(\gamma\delta T\)-cells in the GALT of C2GnT2-deficient mice. Additionally, the list of lectins and glycan binding proteins for which the altered glycan structures in C2GnT2-deficient mice may be ligands is likely to grow, as the glycan specificities for more lectins are determined. However, some of the lectins may not bind to the potential ligands in C2GnT2-deficient mice, as the potential ligands were determined solely based on a carbohydrate structure, and the ability of carbohydrates to interact with lectins is often also influenced by the protein they are attached to. In addition, many of the lectins analyzed for glycan binding specificity by the Consortium for Functional Glycomics are human lectins, and binding specificities for the mouse orthologues may be slightly different.

Analysis of lectins, which may bind to carbohydrate structures that are increased in the mucosa of C2GnT2-deficient mice, also revealed that bacterial lectins and toxins might have altered ability to bind to the mucosa of C2GnT2-deficient mice. Thus, C2GnT2-deficient mice may also have altered susceptibility to various pathogens.
To our knowledge this is the first model found to have a specific reduction in systemic antigen-specific IgGs in response to KLH administered via the oral route but no difference in the humoral response following i.p. immunization. Thus the further study of the systemic and mucosal immune responses in C2GnT2-deficiency may promote a better mechanistic understanding of the immune responses to mucosal antigens and pathogens.

**Materials and Methods**

*Mice*

C2GnT2-deficient mice have been described (Chapter 1). Mice were backcrossed at least 6 generations to the C57BL/6NHsd strain.

*Intraperitoneal immunizations*

Mice were immunized as previously described (Tenno et al., 2007). Briefly, mice were i.p immunized with KLH-DNP (Calbiochem) emulsified with CFA (Sigma) on day 0, followed by a booster immunization with KLH-DNP and IFA (Sigma) on day 28. Every 7 days sera was collected and utilized to measure the relative amount of antigen-specific immunoglobulins of each isotype present in the sera of immunized mice at each time point.
Mucosal immunizations

Mice were perorally immunized as previously described (Vajdy and Lycke, 1993). Briefly, mice were immunized by oral gavage with KLH-DNP plus CT (Sigma) as an adjuvant at time zero and then every 10 days. Sera and stool were collected at time 0 and every 5 days until the completion of the experiment.

Fecal protein solutions

A fecal solution was made as previously described (deVos and Dick, 1991). Briefly, collected feces were stored at -80°C until needed. The feces were then weighed, diluted 20 fold in phosphate buffered saline (PBS), and vortexed every 15 min until all of the feces were in solution.

Determination of relative levels of antigen-specific immunoglobulins

To determine the relative amount antigen-specific immunoglobulins of each isotype in sera ELISAs were performed. Maxisorp 96-well plates (Nunc) were coated with 20 µg of DNP-albumin (Calbiochem) in PBS for 1 hour at 22°C. Plates were blocked with 2% IgG-free BSA (Jackson ImmunoResearch) in PBS for 1 hour at 22°C. Sera samples were diluted 1/200, and allowed to bind for 2 hours at 22°C. Antibodies to murine IgM (Sigma), IgG1, IgG2a, or IgG2b (BD Biosciences) conjugated to alkaline phosphatase diluted 1/2000, or antibodies to murine IgG3 (BD Biosciences) or
IgA (Sigma) conjugated to alkaline phosphatase diluted 1/1000 were allowed to bind for 1 hour 22°C. Para-nitrophenyl phosphate (Sigma) was utilized as a substrate and plates were read at 405 nm.

ELISAs to determine the relative concentration of mucosal antigen-specific IgA were performed with similarly to above with slight modifications. Briefly, fecal solutions were then allowed to bind overnight at 4°C.

_Determination of relative levels of Galectin-1 ligands_

Maxisorp plates were coated with 100 µL of fecal solution overnight at 4°C. Plates were blocked with 2% BSA for 1 hour 22°C. Plates were washed with PBS. Recombinant Galectin-1 (R&D Systems) was biotinylated using NHS-Biotin (Pierce). Briefly, 50 µl of 0.25 µg/µL recombinant Galectin-1 was incubated with 1 µl Biotin-NHS (2.2mg diluted in 400 µl of H2O) for 30 minutes at 22°C, and then the remaining NHS was quenched by incubating with 1µl of 0.1 M Glycine for 30 min at 22°C. This biotinylated-Galectin-1 was diluted 1/1000 in PBS plus 2% BSA and allowed to bind overnight at 4°C. Plates were again washed with PBS and then Streptavidin conjugated to horseradish peroxidase diluted 1/5000 was allowed to bind to plates for 45 min at 22°C. Tetramethylbenzidine was used as a substrate, and plates were read at 650 nm.
**Analysis of cell type frequencies in immune organs**

Immune tissues were isolated and single cell suspensions were made by pushing the tissues through metal screens in FACS buffer (PBS plus 2% bovine serum) using the plunger from a 1 ml syringe (BD). Cells were pelleted and then resuspended in Red Blood Cell Lysis Buffer (eBioscience). Lysis was stopped by with 10 ml of FACS buffer. Cells were then pelleted and suspended in fresh FACS buffer. Cells were counted using trypan blue and a hemocytometer (ICN Biomedicals, Inc.). Cells were then stained in 100 µl of FACS buffer plus 0.5 µl of each antibody (BD Biosciences) and 5 µl of 7AAD (BD Biosciences) to be able to gate on live cells. Binding of various antibodies was determined by flow cytometry (FACSCalibur System, BD Bioscience). Total numbers of a particular cell type was determined by multiplying the number of cells positive for given marker(s) times the total number of cells in the given tissue.
**Tables**

**Table 2.1: Glycan Structures in C2GnT2-Deficient Mice that are Potential Ligands for Lectins and Glycan Binding Proteins.** The table indicates glycan structures that have been identified as potential ligands for lectins that modulate immune response. The structures found to be a potential ligand and examples of O-glycan structures in C2GnT2-deficient mice that contain these potential ligands are shown. This table was partly compiled based on data available on the Consortium for Functional Glycomics website (Smith, 2009).

<table>
<thead>
<tr>
<th>Potential Ligands (Carbohydrate Structures with Altered Expression in C2GnT2-Deficient Mice)</th>
<th>Example of Structure is C2GnT2-Deficient Mice which Includes Potential Ligand</th>
<th>Tissues in Which the Carbohydrate Structure is Altered</th>
<th>Lectins and Glycan Binding Proteins that Bind Potential Ligand</th>
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<tr>
<td><img src="#" alt="Galactose" /></td>
<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>gamma-delta T-cell Receptor (G8 sTCR)</td>
</tr>
<tr>
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<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>DC-SIGN</td>
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<tr>
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<td></td>
<td>CTLY</td>
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<tr>
<td><img src="#" alt="Galactose" /></td>
<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>Collectin K1</td>
</tr>
<tr>
<td><img src="#" alt="Galactose" /></td>
<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>Cholera Toxin Beta Subunit</td>
</tr>
<tr>
<td><img src="#" alt="Galactose" /></td>
<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>Beta-subunit of E. coli Heat-Labile Enteroxin</td>
</tr>
<tr>
<td><img src="#" alt="Galactose" /></td>
<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>BdC Bacterial Lectin</td>
</tr>
<tr>
<td><img src="#" alt="Galactose" /></td>
<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>E. coli Cytolethal Distending Toxin Type III, A subunit</td>
</tr>
<tr>
<td><img src="#" alt="Galactose" /></td>
<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>gamma-delta T-cell Receptor (G8 sTCR)</td>
</tr>
<tr>
<td><img src="#" alt="Galactose" /></td>
<td><img src="#" alt="GalNAc" /></td>
<td></td>
<td>BclB Bacterial Lectin</td>
</tr>
</tbody>
</table>

- **Galactose**
- **GalNAc**
- **Fucose**

*Table 2.1 Glycan Structures in C2GnT2-Deficient Mice that are Potential Ligands for Lectins and Mammalian Binding Proteins*
Figures

Figure 2.1: The humoral response to KLH following i.p. immunization in C2GnT2-deficient mice. Relative sera levels of antigen-specific immunoglobulins of each isotype following i.p. immunization with KLH. Results for wild-type mice are depicted with closed squares and C2GnT2-deficient mice data is graphed with open circles.

Figure 2.2: C2GnT2-deficient mice have a reduced antigen-specific humoral response to KLH as a mucosal antigen. (a) The relative levels of antigen-specific immunoglobulin of each isotype in sera in wild-type (closed squares) and C2GnT2-deficient mice (open circles) following mucosal immunization with KLH. (b) The relative levels of antigen-specific IgA in fecal solutions in wild-type (closed squares) and C2GnT2-deficient mice (open circles) following mucosal immunization with KLH.
Figure 2.3: Expression of Galectin-1 ligands in mucosa of C2GnT2-deficient mice.
Relative levels of ligands for Galectin-1 in fecal solutions from wild-type and C2GnT2-deficient mice are shown. p=0.0554

Figure 2.4: Preliminary analysis of frequency of various cell types in the GALT from C2GnT2-deficient mice. a) Frequency of cell types, based on expression of cell markers, in mesenteric lymph nodes of wild-type and C2GnT2-deficient mice. b) Frequency of cells expressing specific cell surface markers in Peyer’s patches from wild-type and C2GnT2-deficient mice. While the frequency of TCRγδ+ cells was increased in the Peyer’s patches of C2GnT2-deficient mice, there was no significant difference in the total number of TCRγδ+ cells in this number of mice. (**, p<0.01)
Acknowledgments

Chapter 2 in part contains data obtained through collaboration with Mohd Nazri Ismail, Stuart M. Haslam and Anne Dell to determine O-glycan structures. Chapter 2 also contains in part data and modified text from the abstract, background and significance, preliminary studies sections of a grant I co-authored with Jamey D. Marth. I was the primary investigator of the study presented in this chapter.
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Mammalian N-Glycan Branching Protects against Innate Immune Self-Recognition and Inflammation in Autoimmune Disease Pathogenesis

Ryan S. Green,1,2 Erica L. Stone,1,2 Mari Tenno,1,2,3 Eero Lehtonen,1 Marilyn G. Farquhar,1 and Jamey D. Martin1,2,4

1Department of Cellular and Molecular Medicine
2Howard Hughes Medical Institute
University of California, San Diego, La Jolla, CA 92033, USA
3These authors contributed equally to this work.
*Correspondence: jmartin@ucsd.edu
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SUMMARY

Autoimmune diseases are prevalent and often life-threatening syndromes, yet the pathogenic triggers and mechanisms involved remain mostly unresolved. Protein asparagine-linked (N-) glycosylation produces glycans structures that substantially differ among the extracellular compartments of evolutionarily divergent organisms. Alpha-mannosidase-II (α-MII) deficiency diminishes complex-type N-glycan branching in vertebrates and induces an autoimmune disease in mice similar to human systemic lupus erythematosus. We found that disease pathogenesis provoking glomerulonephritis and kidney failure was nonhematopoietic in origin, independent of complement C3 and the adaptive immune system, mitigated by intravenous administration of immunoglobulin-G, and linked to chronic activation of the innate immune system. N-glycans produced in α-MII deficiency bear immune-stimulatory mannoside-dependent ligands for innate immune lectin receptors, disrupting the phylegetic basis of this glycan recognition mechanism. Thus, mammalian N-glycan branching safeguards against the formation of an endogenous immunologic signal of nonsself that can provoke a sterile inflammatory response in the pathogenesis of autoimmune disease.

INTRODUCTION

Autoimmune diseases arise when immune stimuli override mechanisms of self tolerance and are often diagnosed by elevations in autoantibody titers. Pathogenesis is generally attributed to the effector functions of the adaptive immune system. Systemic lupus erythematosus (SLE) is an autoimmune syndrome in which autoantibodies to nuclear antigens and immune complex formation are induced with a high prevalence of kidney disease (Jorgensen et al., 2004; Laurens and Wolkaid, 2005). The etiology of the SLE-like syndrome that develops from the absence of α-MII is puzzling (Chui et al., 2001). α-MII is conserved among mammals, and its deficiency in mice alters protein N-glycosylation in some cell types by blocking the formation of complex-type N-glycans that normally constitute the predominant N-glycan branching structure on the carbohydrate cell surface (Chui et al., 1997).

Incomplete N-glycan branching in the Golgi apparatus results from α-MII deficiency, leading to the appearance of hybrid-type N-glycan structures at the cell surface. As mice age without α-MII function, signs of SLE invariably appear with an increase in anti-nuclear antibody (ANA) titer, a hematologic abnormality characterized as dyserythropoietic anemia, and glomerular deposition of immunoglobulin and complement component C3. Glomerulonephritis is the major pathologic feature in these animals leading to oedema, renal dysfunction, and kidney failure in a syndrome indicative of lupus nephritis (Chui et al., 2001). Remarkably, diminished N-glycan branching is observed among only a subset of cell lineages in α-MII deficiency resulting from the presence of another mannosidase, termed α-MI, that can compensate to promote complex-type N-glycan branching (Chui et al., 1997; Akama et al., 2006). Although this compensation occurs among lymphoid and myeloid cells, it is absent from the erythroid lineage and variable in efficacy among multiple cell types (Chui et al., 1997, 2001; Akama et al., 2006). For example, T and B lymphocytes that lack α-MII retain high amounts of complex-type N-glycans. Not surprisingly, lymphoid cells develop normally in α-MII deficiency and respond to immunologic stimulation without alterations (Chui et al., 2001). These findings have suggested that α-MII deficient autoimmune disease may reflect a distinct etiology encompassing a pathogenic mechanism that involves endogenous epitope modification (Wolkaid et al., 2001).

We have used molecular and cellular approaches that discriminate among the roles of cell lineages to determine the origin and mechanism of the SLE-like autoimmune disease resulting from α-MII deficiency. Our findings show...
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a pathogenic trigger among nonhematopoietic cell types and glycoproteins that undergo altered protein N-glycan branching, indicating a defective signal of infection and inflammation composed of extracellular mannose-dependent N-glycan ligands of innate immune lectin receptors. The adaptive immune system does not appear to play a pathogenic role and instead modifies the disease course, attenuating the inflammatory route that encompasses macrophage recruitment, glomerulonephritis, and kidney failure. Pathogenesis in αM-II deficiency arises by disrupting a mechanism of vertebrate innate discrimination by which the innate immune system normally distinguishes the glycans of lower eukaryotic and prokaryotic organisms.

RESULTS

Normal Kidney Morphogenesis in αM-II Deficiency

Tissue and organ damage in SLE is often focused and most severe in the kidney. To investigate the pathogenic basis of this autoimmune disease syndrome in αM-II deficiency, we first studied kidney development and early postnatal function. Among kidneys of animals analyzed at birth, we observed normal glomerular organization at the electron microscope level with normal glomerular basement membrane, epithelial foot processes, and filtration slits (Figure S1 in the Supplemental Data). We found, in addition, kidney function in mice lacking αM-II was unimpaired during the first 6 months of life as determined by urinary measurements of hematuria and proteinuria. These findings indicate that the development of glomerulonephritis between 6 and 9 months of age is not associated with an ontogenic or functional disruption of the kidney per se.

Nonhematopoietic Origin of Autoimmune Disease in αM-II Deficiency

Autoimmune diseases may be of hematopoietic origin and resolve to abnormalities in lymphoid cell function, as evidenced in numerous bone-marrow transplantation studies (Ikehara, 1998). We subjected 8-week-old wild-type and αM-II null littermates to irradiation levels that were invariably lethal without hematopoietic reconstitution by bone-marrow grafts. At this age, disease pathology is not yet present and all mice are healthy. Syngeneic bone-marrow recipients that were fully reconstituted by donor-derived hematopoietic cells by 8–10 weeks and which retained normal peripheral leukocyte numbers in circulation were further studied up to 9 months after transplantation. Recipients analyzed remained donor derived throughout the course of the study as determined by hematopoietic cell surface N-glycan markers and genomic DNA analyses (Figure 1A and data not shown). No complications reflecting graft-versus-host disease were noted in the experimental populations. Lymphoid lineage development, abundance, and activation responses were unaffected, as in systemic αM-II-deficient mice, because of the presence of the αM-IX isozyme (Chui et al., 2001). Remarkably, elevated autoantibody titers to cellular and nuclear antigens, glomerular deposition of immunoglobulin-G and complement C3, as well as kidney inflammation and dysfunction were linked to αM-II-deficient recipients (Figures 1B–1F).

Autoimmune disease pathogenesis was neither provoked by αM-II-deficient bone-marrow-derived cells nor attenuated by wild-type marrow grafts. Instead, the pathogenic trigger that leads to the induction of autoantibodies and kidney disease in αM-II deficiency resides among nonhematopoietic cell types.

Among disease markers, only the dyserythropoietic anemia was linked to αM-II-deficient bone-marrow-derived and hematopoietic lineages (Figure 1G). This is consistent with the erythroid dependence on αM-II, a cell lineage wherein no compensation by the αM-IX isozyme occurs, resulting in hybrid-type N-glycans at the cell surface and increased turnover of the anisotric population of circulating erythrocytes (Chui et al., 1999). It is evident from these studies that this erythroid defect does not contribute to the elevation of autoantibody titers or to kidney disease.

Attenuation of Disease by the Adaptive Immune System

The adaptive immune system fails to develop without recombinase-activating gene 1 (RAG-1); mature lymphocytes, antibodies, and immune complexes do not exist in such animals (Kobayashi et al., 1991). In the context of αM-II deficiency, it was possible to determine the contribution of the adaptive immune system to disease pathogenesis with a focus on the neutral component. Unexpectedly, mice lacking both αM-II and RAG-1 were more severely affected with loss of weight, alopecia, and exacerbation of kidney disease, as compared with littermates lacking αM-II or RAG-1 alone (Figure 3G). Glomerulonephritis was intensified and a high degree of nephrosis occurred concomitant with hematuria and proteinuria (Figures 2A and 2B).

Molecules produced by lymphocytes that can modulate autoimmune disease include immunoglobulin-G (IgG), which is a ligand for Fc receptors expressed on various cell types (Ninomiya and Ravenst, 2000). Fc receptor function can decrease immune responses. For example, FcγRIIIb attenuates immune activation and mice lacking this Fc receptor spontaneously develop autoimmune disease (Takai et al., 1990). We suspected that the absence of inhibitory signaling through Fc receptors among mice lacking RAG-1 might accelerate inflammation and nephritis in the αM-II null background. Cohorts of animals were given multiple intravenous injections of normal mouse IgG (IVG) for a period of 4–6 months while disease signs were followed. In the absence of both αM-II and RAG-1, IVG therapy diminished disease signs coincident with improved kidney function (Figure 2J). The adaptive immune system therefore was not the source of the pathogenetic trigger, nor was it required for disease progression, but instead appeared to moderate inflammation and glomerulonephritis.
Figure 1. Nonhematopoietic Origin of Autoimmune Disease in αM-III Deficiency

(A) Bone marrow transplantation to reconstitute the hematopoietic system was accomplished among 7-month-old recipients. All indicated times after transplantation, donor and recipient genotypes were detected by enzymozyme cell-surface binding of the erythrocytomegakaryocyte (E-PHA) that binds complex N-glycolipids and is dependent upon αM-III function. Results were similar to hematopoietic cell (PHA-proliferation studies not shown).

(B) αM Autoimmune markers were analyzed routinely for 6-9 months after transplantation, and disease occurred only in recipients that lacked αM-III function. These findings encompassed (B) and nuclear antibody (ANA) titers in sera diluted 1:200 with gG binding titers to nuclear epitopes and IgM.

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Pathogenesis is Independent of C3 Complement
Deposition of C3 complement in the kidney is elevated among glomeruli of sM-II-deficient mice concurrently with decreased amounts of C3 in circulation. Nevertheless, absence of complement C3 in sM-II deficiency failed to inhibit the development of SLE disease markers, including elevated autoantibody titers and nephritis, as well as anemia, indicating that deposition of C3 is not an important pathogenic factor (Figure 3). This finding differs from C3 production and its glomerular deposition from disease pathogenesis in the absence of sM-II. Therefore, C3 deposition tracks with disease signs including glomerulonephritis but does not represent the pathogenic trigger. The persistence of nephritis in the absence of either C3 complement or the adaptive immune system focused attention on the possible role of the innate immune system in disease pathogenesis.

sM-II Deficiency Stimulates the Innate Immune System
We examined the innate immune system in mice lacking sM-II and found that macrophage infiltration in the kidneys is an early and consistent disease marker that is further elevated by the absence of RAG-1 (Figure 4A). NVG treatment reduced macrophage recruitment, coincident with improved kidney tone, appearance, and function. Macrophages lacking sM-II retained normal functions and activation responses when analyzed ex vivo; as might be expected because they can continue to express complex-branched N-glycans ascribed to the sM-II isozyme (Figure S3; Chui et al., 2001; Akama et al., 2008). Macrophages infiltrating the kidneys of sM-II-deficient mice expressed activation markers including elevated MHC class-II expression and were often found proximal to the glomerular capsule and adjacent to mesangial cells (Figure 4B). The abundance of activated macrophages in the kidneys of sM-II-deficient mice was correlated with the degree of inflammation and tissue damage. This relationship extended to the amount of inducible nitric oxide synthase (iNOS) that generates nitric oxide, which is a reactive oxygen species associated with inflammation after an innate immune response and increased glomerular injury in SLE (Tachman, 2004; Oost and Gilkeson, 2009). iNOS expression in the kidneys was evident in sM-II-deficient mice but not among wild-type littermates. Amounts of iNOS were substantially elevated by RAG-1 deficiency and mediated by NVG treatment (Figure 4C).

Mesangial Cells of the Innate Immune System Are Activated in sM-II Deficiency
Mesangial cells in the glomeruli possess innate immune modulatory activity and when activated, they produce cytokines and chemotactic factors such as monocyte chemotactic protein-1 (MCP-1) that recruits and activates macrophages (Matsushima et al., 1993; Laren et al., 1995; Gomezdia et al., 2003). MCP-1 expression has been linked with inflammation and glomerulonephritis in SLE and lupus nephritis (Rovin et al., 1994; Laren et al., 1995; Zoa et al., 1997; Taesch et al., 1998; Kim et al., 2002; Hasegawa et al., 2002; Shimizu et al., 2004; Gomezdia et al., 2003). The activation of mesangial cells appeared early in sM-II-deficient mice with the onset of hyperplasia and glomerular inflammation. Markers of mesangial cell proliferation (Ki-67) and inflammation (IL-1) were prominent by 3–4 months of age. By 8–9 months of age, mesangial cell apoptosis measured by active caspase-3 was consistently observed coincident with increased tissue sclerosis, neprphon loss, and kidney dysfunction (Figure 5A). MCP-1 was absent from wild-type adult kidney tissue at all ages but was induced among mesangial cells of sM-II-deficient littermates (Figure 5B).

binding (red) to membrane and cytoplasmic proteins of HEP-2 cells (700×1), (C) autoantibody titers at 1:40 dilution to kidney-type cellular proteins (including Sm and dsDNA, not shown), (D) glomerular immunoglobulin deposition (160×), as well as kidney dysfunction detected by the frequency of mice exhibiting (E) hematuria and (F) proteinuria after transplantation. In contrast, anemia (G) as detected by low red blood cell (RBC) numbers in circulation occurred in mice transplanted with sM-II-deficient marrow. Autoantibody titers in (C) were calculated as a ratio at the experimental time point (T), divided by the initial dilution (T0). Results are representative and encompass 8 to 16 mice of indicated ages and genotypes. Standard errors of the means are indicated as (C) and (G) as vertical black lines and gray bars, respectively.

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Glomerular expression of MCP-1 was modulated by IVIG treatment, as was the abundance of activated macrophages found adjacent to mesangial cells and the glomerular capsule (Figure 5G). These studies revealed that the activation of mesangial cells in the absence of αM-IL led to the production of proinflammatory proteins that are known pathogenic factors in the etiology of SLE and kidney disease.

**Innate Immune Lectin Receptor Modulation and Proinflammatory Ligand Formation**

Cells of the innate immune system, including mesangial cells and macrophages, express pattern-recognition receptors including lectins that bind to mannose-enriched glycan structures typical of microbial and pathogen cell surfaces (Barton and Mezhbir, 2003; McGreal et al., 2004). For example, the macrophage mannose receptor (MMR) and the mannose-binding lectins (MBL-A and MBL-C in the mouse) bind to glycan structures bearing mannose linkages typical of lower eukaryotic and microbial glycans (Sharon, 1987; Ezekowitz et al., 1990; Sauder et al., 1995; Hansen et al., 2000; Gordon, 2002; McGreal et al., 2004). Mannoselike lectins of the innate immune system therefore appear to distinguish evolutionarily distinct organisms based in part upon unique differences in mannose linkages expressed among cellular glycans. Mesangial cells are exposed to a variety of stimuli that exist in vascular circulation and which enter the kidney glomerulus during normal filtration processes. Although macrophages are recruited and activated by MCP-1, macrophages were not directly stimulated by hybrid N-glycans produced in the absence of αM-IL (Figure 5D). The source of the mesangial cell activation stimulus was investigated among N-glycans in circulation. Serum from wild-type mice induced some MCP-1 production in isolated cultures of glomeruli, whereas the addition of the yeast cell wall constituent mannan induced a significantly greater response. MCP-1 production was also markedly...
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Figure 4. Innate Immune Activation and Kidney Inflammation
(A) Macrophage recruitment was detected by means of anti-COX-2 antibody, and fluorescent signals were quantified among multiple-kidney sections. Results were plotted from five 8-month-old mice of indicated genotypes.
(B) Expression of MHC class II among CD68+ macrophages in the kidneys of αM-II null mice (top, fluorescent colocalization in yellow, effective magnification ×2000x). RCA-1 binds to macrophages (middle) and detected in the glomerulus with mesangial cells adjacent to CD68+ macrophages (bottom, magnification ×500x).
(C) NOX protein expression was measured by fluorescence in kidney sections from mice of the indicated genotypes.
(D) NOX expression was analyzed among various cell types including kidney proximal tubular epithelial cells (in yellow, mAb) and NOX expression on the glomerular mesangial cells (in green, mAb). Data are representative of four independent experiments done in triplicate from mice of the indicated genotypes.

Increased in cultures of isolated glomeruli treated with serum from αM-II-deficient mice. Moreover, this response was that of mannose, was inhibited in the presence of the mannose analog and mannose lectin binding inhibitor alpha-methylmannoside (MMI) (Figure 6A). These findings indicated that N-glycans present in the sera of αM-II-deficient mice induced mesangial cell activation and proinflammatory cytokine production by a mannose-dependent binding mechanism.

Among innate immune lectins that exhibit mannose-binding activity, the expression of the MMR, MBL, A, and MBL-C was analyzed. In αM-II-deficient mice, we observed that MMR protein expression was induced on mesangial cells in the kidney and was present among a proportion of intercellular macrophages (Figure 6B and data not shown). In studies of MBL protein abundance, we noted reduced serum concentrations of both MBL-A and MBL-C that correlated with their increased deposition in the kidney of αM-II-deficient mice (Figures 5C and 5D). The modulation of mannose-binding lectin expression observed in αM-II deficiency implicated the presence of endogenous glycan ligands.

By using recombinant mammalian MMR and MBL chimeras, mannose-dependent ligands of these lectins were detected in the sera of αM-II-deficient mice (Figure 6E). Multiple glycoproteins exhibited ligands that were recognized by both lectins, and this binding was competed by αMM. The presence of endogenous ligands among various cell types was further investigated and revealed accumulation of mannose-dependent MMR ligands primarily among kidney tissue and mesangial cells within the glomerular capsule, but not among various other organs and cell types (Figure 6F). Ligands of MBL-A and MBL-C lectins were also present among glycoproteins
in the kidneys of αM-II deficient mice as well as among glycoproteins expressed on the erythrocyte cell surface (not shown). In further studies, inherited deficiency of the MBL-A lectin with the coexistence of αM-II continued to induce autoimmune disease (Figure S4; and data not shown). The absence of disease modulation by a single lectin deficiency may represent cell-type-specific functions and compensation involving a growing list of mannose-binding lectins expressed among cells of the innate immune system including DC-2D2, Enos163, DC-SIGN, LangRah, Def10, and BDC-1. Our findings have shown that altered N-glycosylation because of loss of αM-II activity led to the production of proinflammatory mannose-dependent glycan ligands for multiple innate immune lectin receptors. These endogenous ligands have structural similarity with glycans linkages on the cell surface of lower eukaryotes and microbes (Figure S5).

**DISCUSSION**

Protein N-glycosylation is altered in αM-II deficiency resulting in the formation of hybrid type N-glycans bearing mannose-dependent ligands of innate immune lectin receptors that induce inflammation. Endogenous ligands of innate immune lectins do not normally accumulate among extracellular compartments of vertebrates, and those produced in the absence of αM-II appear to act as a pathogenic trigger of noninfectious disease consistent with the etiologic features of the resulting SLE-like autoimmune disease syndrome. We found that disease progression tracked with chronic stimulation of the innate immune system involving mesangial cell activation and macrophage recruitment in a sterile milieu lacking an infectious and causative pathogen. Inflammation and cell death in the kidney was accompanied by markers of nitric oxide production and increased antigen presentation, yet neither elevated autoantibody titers nor complement C3 were factors in disease progression, and neither appeared to play a substantial pathogenic role.

The increased severity of kidney disease among animals lacking both αM-II and RAG-1 indicated an etiology independent of peptide antigen presentation mechanisms and the adaptive immune system. On balance, the adaptive immune system provides a modifying effect in disease progression. This moderation does not appear to reflect alterations in lymphoid cell subpopulations including regulatory T cells, which were similar between mutant and wild-type genotypes (data not shown). Among genetic lesions that alter the immune system and induce kidney disease, a lymphocyte independent mechanism of pathogenesis has been similarly detected by loss of RAG-1 function among mice lacking the Lyn tyrosine kinase (Yu et al., 2001). Lyn deficiency accelerated autoimmune disease symptoms induced by the absence of the Lyn tyrosine kinase, appearing to reflect a kidney intrinsic role of Fyn, SLE-like disease signs induced by αM-II deficiency followed normal kidney development and early postnatal function and may be augmented by immunologic disease processes similarly engendered by other genetic lesions.

Administration of IVIG in αM-II deficiency reduced macrophage recruitment and disease severity evoked by RAG-1 cedeiciency, providing the moderating effect.
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ascribed to the adaptive immune system. Autoantibodies detected in Sm-II deficiency do not exhibit N-glycan binding (Chiu et al., 2001), implying that conserved regions within the IgG molecule participate in disease attenuation. IVIG is a therapeutic treatment in human diseases of inflammation and autoimmunity including myasthenia gravis, Guillain-Barré syndrome, graft-versus-host disease, Kawasaki's syndrome, as well as SLE, in which it may operate by promoting inhibitory signaling of Fc receptors (Off et al., 2001; Kasahara and Kawan, 2001; Sheiner and Sheinold, 2006; Park Min et al., 2002). It is possible that loss of FcγRIIb in the Sm-II deficient background may also elevate innate immune inflammatory responses and increase severity. Our findings support the view that the therapeutic efficacy of IVIG treatment reflects at least in part a reduction in innate immune activation, and we suggest that genetic or spontaneous variation in human protein N-glycan branching frequency may also alter IVIG efficacy in disease intervention.

The nonhematopoietic origin of innate immune activation and autoimmunity in Sm-II deficiency can be further resolved into a molecular and pathogenic trigger. Alterations in the functions of N-glycoproteins that normally regulate the innate immune system remain possible but unlikely, because inflammation did not appear systemically and immune cell response parameters were unaffected by the absence of Sm-II. Moreover, a pathogenic trigger was detected among the hybrid N-glycans bearing exposed mannose moieties resulting from the loss of Sm-II function. These pathogenic N-glycans reside on circulating glycoproteins and perhaps immune complexes that macrophages bearing MIF as an N-terminus detect, and may then induce MCF-1 production and an organ-selective disease process. The nonhematopoietic and cellular origin of these ligands may include various cell types that secrete N-glycoproteins into circulation and may also involve the kidney should some cells within this organ lack sufficient compensation afforded by expression of the Sm-Ill isoform.

Innate immune mannose-binding lectins, of the C-type family, include endocytic and phagocytic receptors that promote foreign antigen uptake, intracellular processing, and antigen presentation (McGreal et al., 2004; Robinson et al., 2005). At least some stimulate intracellular signaling pathways involving tyrosine kinases and phosphatases (Lopez-Hernsa et al., 2005; Robinson et al., 2005; Sheng et al., 2005). The stimulation of MIF-1 production from infected glomeruli by Sm-II deficient sera was blocked by SmII, implying that a mannose-dependent binding event induces inflammatory signaling by macrophages. Elevated expression of MIF-1 is common in glomerulonephritis among humans and mice wherein this cytokine recruits and activates macrophages (Matsuzaka et al., 1986; Largen et al., 1995; González-Guerrero et al., 2003). Moreover, MIF-1 is linked to pathogenesis and nephritis in some autoimmune syndromes (Rovin et al., 1993; Largen et al., 1999; Zoja et al., 1997; Teich et al., 1993; Kim et al., 2002; Hasegawa et al., 2003; Shirahata et al., 2004; González-Guerrero et al., 2003).

Ligands of C-type lectins with mannose-binding activity have been proposed to consist of repetitive arrays of terminal mannose residues that normally reside among pathogen-derived oligomannose structures. A significant but limited degree of molecular spacing among mannose linkages is believed to be important in pathogen recognition by facilitating lectin domain interactions (McGreal et al., 2004). Nevertheless, low levels of endogenous ligands exist among few mammalian glycoproteins including the glycosylated proteins that are secreted in inflammation (Stohl et al., 1999; Sheinold et al., 1995). Mannose glycoproteins that are trafficked to the lysosomes by the mannose-6-phosphate signal are also processed by Sm-II (Nink and Kornfeld, 1980), and therefore such N-glycoproteins display mannose linkages also present in the absence of Sm-II. Nevertheless, substantial binding specificity is evident among mannose-binding lectins of the mammalian innate immune system that normally precludes extensive self-recognition.

Although erythrocytes bear hybrid N-glycans at their cell surface in Sm-II deficiency that are also endogenous MBL and MMR ligands, they do not contribute to kidney disease or to the elevation of autoantibody titers. This may reflect a lesser degree of coaggregation involving exposed mannose linkages among N-glycoproteins when tethered to the cell surface or perhaps the sequestration of erythrocyte accumulation and turnover in the spleen. Interestingly, Sm-II deficient mice bear an increased number of splenic macrophages concurrent with an elevation in circulating monocyte numbers in the blood. Although humans deficient in Sm-II activity may exist among some cases of congenital dyserythropoietic anemia type II (also known as HEMAPS), genetic linkage studies of this syndrome have excluded the Sm-II gene (Ishidias et al., 1997; Lanzara et al., 2003). Clinical diagnosis is inexact at present, and whether N-glycosylation defects associated with human HEMAPS are identical to those induced in the absence of Sm-II, extend to all cell types, and provoke autoimmune disease as these patients age remains to be established.

The phylogenetic and structural contexts of mannose linkages are important. Although a portion of total cellular N-glycans in the Golgi of wild type mammalian cells are hybrid N-glycans, these are predominantly synthetic intermediates and do not normally reach the cell surface in substantial amounts. In phylogeny, vertebrates are unique as compared with invertebrates and lower eukaryotes in the mannose linkages exposed among extracellular glycans. Those high-mannose N-glycans found on mature glycoproteins in vertebrates, as well as those on budding viruses, typically bear terminal α2,6 mannose linkages mimicking the underlying α3 and α6 mannose linkages of the core N-glycan structure. The fraction of N-glycans with α2,6-linked terminal mannoses is unabated by Sm-II deficiency (Chiu et al., 2001). Instead, extracellular hybrid-type N-glycans bearing terminal α3 and α6 mannose linkages are produced at the expense of complex-type N-glycan branching, mimicking structural features among the exposed terminal mannose linkages typical of...
Figure 6. Mannose-Binding Lectin Expression and Endogenous Ligand Formation

(A) MCP-1 production among isolated glomeruli after incubation with sera from MMR+/- and MMR-/- 9-month-old mice of diethylnitrosamine (oMM) and WT sera. Differences observed between MMR-/- and WT sera with or without pretreatment with 1 mg/ml of alpha-methylmannoside (oMM) were significant (p = 0.039 and p = 0.018, respectively). MCP-1 levels in culture upon addition of mouse sera and FCS at 0 to 10 were 2 pg/ml or less. Data shown are the mean and standard error from six or more independent experiments with each condition.

(B) Macrophage mannose receptor (MR) expression among mesangial cells in kidney sections (RCA-1) (co-localization in yellow, 400x). Large panels at left are magnified 760x.

(C) Abnormalities of alpha-mannosidase (MMR) activity and MBL levels in the sera of mice of indicated genotypes. Serum from mice of indicated genotypes were evaluated.

(D) Histological analysis of kidney sections from MMR+/- and MMR-/- 9-month-old mice of the indicated genotypes (400x).

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of invertebrates and many lower organisms. Among yeast mutants comprised of differing proportions of mannosic linkages, the α1,3 and especially the α1,6 linkages are the most potent in assays of mannose receptor ligand binding and glycoprotein clearance (Stahl et al., 1998).

The SLE-like syndrome that develops in αM II deficiency originates from an endogenous nonhematopoietic and organ selective pathogenic stimulus composed of hybrid-type N-glycans that promote sterile inflammation, leading to the elevation of systemic autoimmune antibodies, cell activation and apoptotic death, and kidney failure. Autoimmune disorders such as Muckle-Wells and familial cold urticaria bear some pathologic similarities, because they also arise from the dysfunction of endogenous cellular components (Gutterman et al., 2006). Although the disease trigger in αM II deficiency appears to mimic a foreign entity, our findings are consistent with a pathogenic mechanism of autoimmune disease in which endogenous cellular signals of danger and infection may contribute in the absence of an exogenous pathogenic stimulus (Matzinger, 2002).

Other glycans from various sources that include chitin, N-acetylglucosamine residues, and fragments of hyaluronic also activate the innate immune system and may in some cases contribute to disease pathogenesis (Maldonado et al., 1995; Ji et al., 1996; Reuss et al., 2007). We infer that the development of complex αM glycans branching during vertebrate evolution in part enabled the immune system to acquire and retain lectin receptors to detect and signal the presence of non-self upon encountering the extracellular glycans of potential pathogens. This interaction may be exploitably in immunization protocols should terminal α1,3 and α1,6 mannosic linkages provide an adjuvant function that explains the heightened immune responses to some N-glycosylated antigens synthesized in yeast and mammalian cells. The transfer of mammalian N-glycosylation pathways into microbial organisms may usurp this mechanism of non-self discrimination and perhaps alter the outcome of infection. Transcriptional networks and mutagenic events that disperse the formation of complex αM glycans branching among mammals can induce sterile inflammation and may serve as the pathogenic bases of some autoimmune and inflammatory diseases.

**EXPERIMENTAL PROCEDURES**

**Mice**

Snell included (C57/10-H-1 [DA], X 1991), H-2d (C57Bl/6J), FR-1 (C57Bl/6J, C3H/HeJ, BALB/c), and DBA (C57Bl/6J) were used. For experiments, at least 21 mice were identified by the detection of a cell line in the presence of the above-mentioned cell lines, 72 h after injection. Mice were inoculated via the tail veins with 10⁶ bone marrow cells or 500 ul of phosphate-buffered saline (PBS). In addition to TCR analyses of spleen or peritoneal cavity (Boy et al., 1996), discrimination of the stem, germine DNA from bone marrow cells of recipients (Purkayastha, 1997) was analyzed by polymerase chain reaction.

**Bone-Marrow Transplantation**

Recipient mice of each relevant genotype were administered 10-11 Gy of gamma radiation from a Cs137 source of 8-14 weeks of age for spleen transplantation experiment (Conley et al., 2005). Donor cells were isolated from bone marrow of 8-10-week-old mice. At 3 weeks after irradiation, recipient mice were injected with 10⁵ bone marrow cells or 500 μl of phosphate-buffered saline (PBS). In 3 weeks, we analyzed the TCR repertoire of spleen or peritoneal cavity (Boy et al., 1996). Donor mice were analyzed by polymerase chain reaction.
phosphate (Sigma) for 15 min at 25°C, and reactions were stopped with 60 μl of 0.1 M EDTA. Samples were measured with a Ventracel microplate reader at 450 nm ( Molecular Devices Corporation, Sunnyvale, CA). For HRP-conjugated antibody, plates were developed by adding 100 μl of TMB (tetramethylbenzidine) (YMB) substrate solution (Sigma) and signals measured at 650 nm.

To detect solid-phase antibody (ANA) assays were performed as described (Zaika et al., 1991) with no modification. For each reaction antibody extraction, sera from mice were diluted 1:100 in PBS and incubated with 1:1000 dilutions of anti-ANA conjugated HRP or PTTh-conjugated anti-IgM (American Research, West Grove, PA) at 1:480 for 30 min at 25°C. Plates were washed in PBS for 20 min, and antibody binding was detected with the peroxidase-conjugated anti-ANA or PTTh-conjugated anti-IgM (American Research, West Grove, PA) at 1:480 for 30 min at 25°C. Plates were washed in PBS and mounted with counterstain for fluorescent microscopy with a Zeiss Axioskop fluorescent microscope (Zeiss, Gottingen, Germany).

Kidney Histology and Function

Tissues were fixed in 4% formalin, decalcified in 7% (v/v) NaOCl (Sigma) and embedded in paraffin. Serial sections were cut at 5-μm thickness and stained with hematoxylin and eosin (H&E) to assess kidney function. To detect antibody infiltration, kidneys were fixed in 4% (v/v) formaldehyde in PBS and mounted. Tissue sections were stained with counterstain for fluorescent microscopy with a Zeiss Axioskop fluorescent microscope (Zeiss, Gottingen, Germany) and observed with a Zeiss Axioskop fluorescent microscope (Zeiss, Gottingen, Germany). Images were obtained with a Photomicroscopy CoolSNAP ES CCD camera (Research Electronics International, Tuscon, AZ) and analyzed by ImageMaster (Cambridge, CA). All experiments were performed using H&E-stained sections of the kidneys.

Macrophage Infiltration and Activation Assays

Isolation of macrophages from mice was performed as previously described (Lauda et al., 1995). Peritoneal macrophages were isolated from BALB/c mice by peritoneal lavage as described. Cells were obtained from the peritoneum of mice and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS). After 24 h of culture, the cells were harvested and stained with FITC-conjugated anti-mouse CD11b antibodies. The percentage of CD11b+ cells was determined by flow cytometry.

Lectin and Ligand Analyses

For analysis of lectin binding, 293 cells were cultured in 10-cm dishes and stained with fluorescein-labeled lectin. Lectin binding was determined using a flow cytometer (FACSCalibur, BD Biosciences). The percentage of lectin-positive cells was determined by flow cytometry.

Statistical Methods

Unless otherwise indicated, Student’s t-test was performed to determine statistical significance.
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Supplemental Data

Mammalian N-Glycan Branching Protects against
Innate Immune Self-Recognition and Inflammation
in Autoimmune Disease Pathogenesis
Ryan S. Green, Erica L. Stone, Mari Tenno, and James D. Marth

Supplemental Experimental Procedures
The kidneys were fixed in 1.5% glutaraldehyde, 3% paraformaldehyde, 5% sucrose in 0.1M cacodylate buffer, pH 7.4, for 1h at RT followed by overnight at 4°C. Post-fixation with 1% OsO₄ in the same buffer for 1hr., then on-blue staining in 1% uranyl acetate in 10% ethanol for 1hr, dehydration in ethanol, embedding in LR112. Semi-thin sections (0.5 µm) were stained with toluidine blue and thin sections with uranyl acetate and lead citrate. The electron microscope was a Jeol JEM 1200EX II.

Supplemental Reference
Figure S1. Kidney Development among αM-II Null Mice
Glomeruli of 2-day old mice. (A,C,E) WT and (B,D,F) αM-II null littermates were analyzed by light microscopy (A, B). The paracortical zone and shows rather mature adult-type glomeruli. The cellularity and overall morphology of the glomeruli are similar in mice of both genotypes. Using transmission electron microscopy (C,D,E,F), the general morphology of the glomeruli is normal and similar in (C) WT and (D) αM-II null kidneys. The ultrafiltration apparatus is normal in both genotypes, as indicated by regular glomerular basement membrane (GBM) and podocyte foot process (FP) organization including slit diaphragms (E,F, arrowheads). Also evident are red blood cell (RBC), podocytes (P) and endothelial cells (EC).
Figure S2. Physical Appearance and Kidney Mass among Mice of Indicated Genotypes

Mice of indicated genotypes among littermates 6-9 months of age were visually observed for alopecia (hair loss) and kidney mass measurements. These findings are representative of 6-24 mice of indicated genotypes and include tissues analyzed in Figures 2 and 4.
Figure S3. Macrophage Activation and Innate Immune Responses

Littermates of indicated genotypes were studied among mice 6-8 months of age. Analyses encompassed measurements of (A) αM-II-null macrophage activation assessed by nitric oxide production, (B, C) innate immune responses to LPS, and (D) wild-type macrophage activation to various stimuli including red blood cells (RBCs), sera, and kidney tissue glycoproteins (kid pro) from mice lacking αM-II. Error bars indicate standard error of the mean.
Figure S4. **Autoimmune Disease Pathogenesis in the Absence of MBL-A and αM-II**

Littermates of indicated genotypes were studied among mice 6-8 months of age. Analyses encompassed measurements of autoimmune disease signs that included histological detection of immunoglobulin deposition (400X), antinuclear antibody (ANA) assays revealing IgG reactivity primarily to nuclear epitopes (blue) and IgM reactivity primarily to membrane and cytosolic epitopes (red) among HEp-2 cells (400X), anemia (RBC counts), as well as kidney dysfunction by urinalysis using measurements of hematuria and proteinuria. Macrophage recruitment to the kidney persisted in mice lacking both αM-II and MBL-A (not shown).
**Figure S5. A Phylogenetic Basis of N-Glycan Branching and Inmate Immune Recognition**

All eukaryotic organisms synthesize the indicated dolichol-oligosaccharide structure for the N-glycosylation of proteins (far left). Although glucose residues (filled circles) are removed during early protein folding and maturation, lower eukaryotes including yeast and fungi subsequently add multiple mannose linkages to N-glycans as they transit the secretory pathway to reside at the cell surface and among extracellular compartments. Invertebrate high-mannose N-glycans can undergo hybrid-type N-glycan branching in constructing N-glycan structures expressed at the cell surface. While structurally distinct from those of yeast and fungi, mature cell surface N-glycans among invertebrates also exhibit terminal α2, α3, and α6 mannose linkages. In contrast, vertebrates primarily produce complex-type N-glycan branching that masks underlying α3 and α6 mannose linkages. High-mannose N-glycans that are secreted are predominantly terminated with α2 mannose linkages. This reflects the presence of Golgi αM-II and N-acetylgalactosaminyltransferase-II (GnT-II) enzymes in vertebrates, resulting in the processing of hybrid-type N-glycans bearing terminal α3 and α6 mannose linkages into mature complex-branched forms. The dotted line represents those vertebrate N-glycan structures rarely found at the cell surface and among extracellular compartments. Absence of the GnT-2 glycosyltransferase in mice has also been found to induce elevated autoantibody titers, glomerular Ig deposition, and kidney inflammation (Wang et al, 2001).
Acknowledgements

Chapter 3, in full, is a reprint of the material as it appears in Immunity:
FUTURE DIRECTIONS

Abstract

The glycosyltransferase Core 2 \( \beta-1,6-N \)-acetylglucosaminyltransferase2 (C2GnT2) is required for generating a normal repertoire of O-glycan structures attached to glycoproteins in the mucosa (Table 1.I). We observed that mice lacking C2GnT2 have reduced levels of circulating and mucosal immunoglobulins (Figure 1.5), but develop a normal antigen-specific humoral response following parenteral immunization with the Keyhole Limpet Hemocyanin (KLH) (Figure 2.1). However, C2GnT2-deficient mice exhibit reduced systemic humoral responses when perorally immunized with the same antigen (Figure 2.2). This unexpected phenotype leads us to believe that further study of the systemic and mucosal immune responses in C2GnT2-deficiency may promote a better mechanistic understanding of the immune responses to mucosal antigens and pathogens. Studies to achieve this goal outlined herein include \textit{in vitro} and \textit{in vivo} analyses to elucidate the cellular and mechanistic bases of this immunologic phenotype. Knowledge gained may ultimately lead to the development of new therapeutics for mucosal infections and pathologies, as well as to improved designs of mucosal vaccines that have the advantages of preventing and protecting from infections including influenza.
Specific Aims

We aim to determine the impact of O-glycosylation on the mucosal immune system. These aims are approached using a mouse model that was rendered deficient for the glycosyltransferase Core 2 $\beta$-1,6-$N$-acetylgalactosaminyltransferase (C2GnT2) using Cre-loxP conditional mutagenesis. This mouse strain is a novel model system in which to study mucosal immunology, as preliminary results show that these mice have a specific defect in the mucosal immune system, but not the systemic immune system, that leads to decreased humoral responses (Chapter 2). The Specific Aims serve to exploit these recent findings to understand how systemic and mucosal humoral responses to mucosal antigens and pathogens are regulated by mammalian O-glycosylation, and to evaluate the effect of altered immunoglobulin expression during a mucosal infection. Increased knowledge in these areas may be beneficial in design of new mucosal vaccines. Further understanding of the mucosal immune system and the development of new mucosal vaccines will have a tremendous impact on human health as 90% of all infections enter through the mucosal surfaces, including most infections with human immunodeficiency virus, effacing and attaching pathogens, and influenza.
Specific Aim 1: Further define systemic and mucosal immune responses in C2GnT2-deficient mice.

a) Characterize the systemic and mucosal humoral responses following intraperitoneal (i.p.) and oral immunizations of C2GnT2-deficient and wild-type mice with ovalbumin (OVA) as an antigen admixed in adjuvant.

b) Determine if oral tolerance is normally induced in C2GnT2-deficient mice upon ingestion of OVA.

c) Compare the ability of Cholera Toxin (CT) to serve as an adjuvant for i.p. administered antigens in C2GnT2-deficient and wild-type mice by immunizing mice of both genotypes with KLH plus CT and comparing the humoral response.

d) Determine the humoral responses to CT as parenteral and mucosal antigen in C2GnT2-deficient mice.

Specific Aim 2: Further understand the mechanistic basis of the reduced humoral response to mucosal antigens in mice lacking C2GnT2.

a) Further compare the frequency of mucosal leukocytes in C2GnT2-deficient mice and wild-type littermates.

b) Analyze germinal center formation in the Peyer’s patches and mesenteric lymph nodes following mucosal immunization of C2GnT2-deficient and wild-type control mice.
c) Determine if *in vivo* and *in vitro* stimulation with CT induces normal maturation of mucosal dendritic cells (DCs) from C2GnT2-deficient mice as determined by expression levels of co-stimulatory molecules, chemokine receptors, and cytokines including major histocompatibility complex class II (MHC Class II), C-C Chemokine Receptor 7 (CCR7), and Interleukin 12 (IL-12), respectively.

d) Analyze cytokine levels including Interleukin-4 (IL-4), Interferon-gamma (IFN\(\gamma\)), and A Proliferation-Inducing ligand (APRIL) following i.p. and oral immunizations of C2GnT2-deficient and wild-type mice.

e) Determine the ability of C2GnT2-deficient B-cells from the mesenteric lymph nodes and Peyer’s patches to undergo isotype switching in the *in vitro* Class Switch Recombination assay.

f) Determine the ability of mucosal T-lymphocytes from C2GnT2-deficient mice to activate and proliferate *in vitro*, and analyze the ability of these T-lymphocytes to activate B-lymphocytes.

g) Analyze intracellular signaling, including phosphorylation of Muc1 and expression of T-bet, in intestinal epithelial cells and mucosal leukocytes from naïve and immunized C2GnT2-deficient mice.

h) Analyze O-glycan structures on intestinal epithelial cells and mucosal leukocytes that can serve as ligands for lectins, including galectins, by immunofluorescence and lectin blots.
i) Produce mice strains deficient in C2GnT2 specifically in hematopoietic or epithelial cell lineages, and determine the humoral response to KLH administered via the mucosal route and the mucosal barrier function in these mice.

Specific Aim 3: Investigate how loss of C2GnT2 modulates mucosal infections using *Citrobacter rodentium* as a model mucosal pathogen.

a) Investigate the course of C. rodentium infection in C2GnT2-deficient mice and wild-type littermates including: survival, ability to clear the infection, and pathology caused by the pathogen.

b) Compare the systemic humoral response in C2GnT2-deficient and wild-type mice following mucosal infection with C. rodentium, to determine if the response is generated by the systemic or mucosal immune system.

Research Design and Methods

Specific Aim 1: Further define systemic and mucosal immune responses in C2GnT2-deficient mice.

The rationale for this aim is to further expand on our preliminary results that C2GnT2-deficient mice have a reduced systemic humoral response to the antigen KLH following mucosal immunization, but not following i.p. immunization. To further understand the role of C2GnT2 in regulating the mucosal immune response, it is necessary to characterize the humoral
response of C2GnT2-deficient mice to additional antigens. As different adjuvants are traditionally used for oral and i.p. immunizations (CT and CFA respectively), it is necessary to confirm that the differences observed in the humoral response following the two immunizations are not specific to the adjuvants used for the immunizations.

a) Characterize the systemic and mucosal humoral responses following intraperitoneal (i.p.) and oral immunizations of C2GnT2-deficient and wild-type mice with ovalbumin (OVA) as an antigen admixed in adjuvant.

C2GnT2-deficient mice and wild-type littermate mice will be i.p. immunized on day zero with OVA-DNP emulsified with CFA, and a booster immunization with OVA-DNP emulsified with IFA will be injected on day 28. Sera will be collected every 7 days through the completion of the study on day 42. For oral immunizations, C2GnT2-deficient mice and wild-type littermates will be administered OVA plus CT as an adjuvant by gavage at time zero and then every 10 days through 45 days (Vajdy et al., 1995). Stool and sera will be collected at time zero and then every five days until the conclusion of the time course. Sera and stool samples will be stored at -20°C and -80°C, respectively. A fecal solution will be made from the stool samples, using 20-fold excess PBS (deVos and Dick, 1991). The fecal solution and sera will then be examined by ELISA to determine the relative amount of antigen-specific immunoglobulin of each isotype. ELISAs will employ immobilized DNP
conjugated to bovine serum albumin (BSA) and probed with anti-mouse immunoglobulin isotype specific antibodies. CFA, IFA, CT, and other potential biohazards will be used in accordance to the policies and procedures outlined by Environmental Health and Safety at UC San Diego.

b) **Determine if oral tolerance is normally induced in C2GnT2-deficient mice upon ingestion of OVA.**

C2GnT2-deficient mice and wild-type littermates will be fed low (1 mg) or high doses (10 mg) of OVA in the absence of adjuvant daily for three days (Barone et al., 1998). One week after the last feeding of OVA, mice will be challenged i.p. with OVA. The response to this challenge will be determined by measuring the *in vitro* proliferative response of lymphocytes as determined by Thymidine incorporation. The humoral response to this OVA stimulation will also be determined by ELISA as in Specific Aim 1a. Additionally, to determine if C2GnT2-deficient mice have a modulated immune response to the normal flora, we will utilize an ELISA-based approach. Cecal flora will be isolated from unimmunized mice (Ohman et al., 2005). This flora will then be immobilized, and immunoglobulins reactive to this flora in the sera of mice will be probed using an enzyme-conjugated antibody to mouse immunoglobulin.
c) Compare the ability of CT to serve as an adjuvant for i.p. administered antigens in C2GnT2-deficient and wild-type mice by immunizing mice of both genotypes with Keyhole Limpet Hemocyanin (KLH) plus Cholera toxin (CT) and comparing the humoral response.

Intraperitoneal immunizations of C2GnT2-deficient mice and wild-type littermates with DNP-KLH and CT will be done on days zero and 10 (Gardby et al., 2003). Sera will be collected from the experimental mice at time zero and then every 5 days for 20 days and stored at -20 until the completion of the experiment. Relative amounts of antigen-specific (DNP) immunoglobulin of each isotype produced though out the experiment will be determined by ELISA as in Specific Aim 1a.

d) Determine the humoral responses to CT as parenteral and mucosal antigen in C2GnT2-deficient mice.

Some mouse models with reduced humoral response following mucosal immunization with protein antigens are still able to generate a normal CT-specific humoral response following immunization. To determine if C2GnT2-deficient mice generate a normal CT-specific humoral response, separate sets of C2GnT2-deficient mice and wild-type littermates will be immunized via the i.p. or oral routes with CT. Immunizations will be done on day zero and then every tenth day for 4 immunizations (Vajdy et al., 1995). Sera will be collected from all mice every five days, and stool will also be collected from perorally
immunized mice. Stool and sera will be stored at -80°C and -20°C, respectively, until the completion of the experiment. The relative amount of anti-CT immunoglobulins of each isotype produced throughout the course of the experiment will be determined by ELISA.

Specific Aim 2: Further understand the mechanistic basis of the reduced humoral response to mucosal antigens in mice lacking C2GnT2.

The rationale for Specific Aim 2 is that further understanding of the mechanistic basis for the reduction of the systemic humoral response to mucosal antigens but not parenteral antigens in C2GnT2-deficient mice will lead to a better understanding of how a systemic humoral response is generated to mucosal antigens. This increased understanding of the differences in the mechanisms used to generate the systemic humoral response to mucosal as opposed to parenteral antigens may lead to the development of better mucosal vaccines.

a) Further compare the frequency of mucosal leukocytes in C2GnT2-deficient mice and wild-type littermates.

Glycosylation has previously been shown to be important for the function and survival of various leukocytes (Ohtsubo and Marth, 2006; Van Dyken, 2007). Thus further analysis of mucosal leukocyte frequencies in C2GnT2-deficient mice (Figure 2.4) will be accomplished. Intraepithelial
lymphocytes and leukocytes from the mesenteric lymph nodes and Peyer’s patches will be isolated. These leukocytes will then be counted using a hemocytometer, and frequencies of specific leukocytes present in each population will be determined using cell-type specific markers characterized by flow cytometry (Tenno et al., 2007). Comparisons to splenic leukocyte populations will allow us to determine if any differences seen are specific to the mucosal immune system.

b) Analyze germinal center formation in the Peyer’s patches and mesenteric lymph nodes following mucosal immunization of C2GnT2-deficient and wild-type control mice.

The observed decrease in specific antigen-specific immunoglobulin isotypes, but not IgM (Figure 2.2), suggests that there is some defect in the mucosa of C2GnT2-deficient mice that leads to a reduction in switched B-cells. Since B-cell switching occurs in germinal centers, it is necessary to determine if germinal centers form correctly in the mucosa of C2GnT2-deficient mice following mucosal immunization. To analyze germinal center formation in the Gut associated lymphoid tissue (GALT) of C2GnT2-deficient mice, mice will be perorally immunized with KLH plus CT. Seven days following immunization, mice will be sacrificed and germinal center formation will be characterized in the Peyer’s patches, mesenteric lymph nodes and spleen of C2GnT2-deficient mice in comparison to wild-types. Germinal
center formation will be analyzed by histology and flow cytometry using T- and B-cell activation antigen GL7 (GL7), and peanut agglutinin (PNA) as germinal center markers (Tenno et al., 2007). In addition, the levels of the co-stimulatory molecules CD40, CD40 ligand (CD40L), and CD86 (B7.2) in germinal center will be determined.

c) **Determine if in vivo and in vitro stimulation with CT induces normal maturation of mucosal dendritic cells (DCs) from C2GnT2-deficient mice as determined by expression levels of co-stimulatory molecules, chemokine receptors, and cytokines including major histocompatibility complex class II (MHC Class II), C-C Chemokine Receptor 7 (CCR7), and Interleukin 12 (IL-12), respectively.**

Stimulation of DCs with cholera toxin leads to the maturation of DCs. Mature DCs have modulated expression of co-stimulatory molecules, cytokines, and chemokine receptors (Gagliardi et al., 2000). Thus to analyze the ability of DCs from C2GnT2-deficient to mature normally following immunization, mice will be immunized perorally with CT and levels of MHC Class II, CCR7 and IL-12 on mucosal DCs will be determined by flow cytometry. Additionally, to determine if any difference seen in vivo is due to a DC intrinsic defect or possibly due to another cell type which interacts with DCs, the expression of the same markers on DCs will be determined following in vitro stimulation of DCs with CT.
d) Analyze cytokine levels including Interleukin-4 (IL-4), Interferon-gamma (IFNγ), and A Proliferation-Inducing ligand (APRIL) following i.p. and oral immunizations of C2GnT2-deficient and wild-type mice.

Cytokines control proper isotype switching, thus to further understand the reason for the decreased levels of specific immunoglobulin isotypes it is necessary to determine cytokine levels in the mucosa of C2GnT2-deficient mice following mucosal immunization. To analyze cytokine levels in response to mucosal immunization, C2GnT2-deficient mice and wild-type littermates will be immunized perorally with KLH plus CT and cells from the mesenteric lymph nodes and Peyer’s patches will be isolated. Intracellular cytokine staining in the isolated cells will be assessed by flow cytometry. To amplify the signal Brefeldin A may be used (Foster et al., 2007). As an alternate method, cytokine levels in the GALT following mucosal immunization will be examined by histology. As a control for both methods, cytokine levels in the spleen will be determined following i.p. immunization.

e) Determine the ability of C2GnT2-deficient B-cells from the mesenteric lymph nodes and Peyer’s patches to undergo isotype switching in the in vitro Class Switch Recombination assay.

As there is a decrease in antigen-specific immunoglobulins following mucosal immunization, it is important to determine if the effect is B-cell intrinsic. One way to investigate this is to isolate B-cells, and determine the
ability of B-cells from GALT of C2GnT2-deficient mice to switch \textit{in vitro} when stimulated with anti-CD40, anti-IgM, or LPS in the presence or absence of IL4. The ability of B-cells to switch to each isotype and to develop into plasma cells will be determined by flow cytometry using antibodies specific to each isotype and Syndecan-1 (CD138), as a marker for differentiation into plasma cells. Cells will be labeled with Carboxyfluorescein succinimidyl ester (CSFE) to determine if switched cells proliferate (Omori et al., 2006). The ability of splenic B-cells to switch \textit{in vitro} will be assessed to determine if any alteration is GALT specific.

\textit{f) Determine the ability of mucosal T-lymphocytes from C2GnT2-deficient mice to activate and proliferate \textit{in vitro}, and analyze the ability of these T-lymphocytes to activate B-lymphocytes.}

Activation of T-cells alters the expression of Core 2 O-glycans on their cell surface, thus the ability of mucosal T-cells from C2GnT2-deficient mice to activate may be altered. It is also necessary to determine if these T-cells are able to activate B-cells normally. Thus to determine if C2GnT2-deficient T-cells activate normally \textit{in vitro} in response to stimulation, CD4$^+$ and CD8$^+$ T-cells from the mucosa of C2GnT2-deficient mice will be isolated, purified, and activated with plate bound anti-CD3 or concanavalin A (ConA) in the presence and absence of IL-2. At 24 and 48 hours after activation, the levels of surface makers including CD69, CD40 and the 1B11 antigen (a monoclonal antibody
that binds in a core 2 dependent manner) on CD4+ and CD8+ T-cells will be determined by flow cytometry. Also, Thymidine incorporation will be assessed to determine if these cells proliferate normally after stimulation. To determine if activated C2GnT2-deficient mucosal T-cells are able to normally activate wild-type and C2GnT2-deficient mucosal B-cells, mucosal B-cells will be added to culture after the activation of mucosal T-cells and then the levels of B-cell surface markers including CD69 and CD86 will be determined by flow cytometry (altered from Tsuboi and Fukuda, 1998).

g) Analyze intracellular signaling, including phosphorylation of Muc1 and expression of T-bet, in intestinal epithelial cells and mucosal leukocytes from naïve and immunized C2GnT2-deficient mice.

While in vitro assays for leukocyte function are important and often informative, it is necessary to determine if these cells function properly in their natural environment, especially considering that intestinal epithelial cells have been shown to be able to modulate mucosal immune function. One way to determine if cells are responding normally to stimuli in vivo is to analyze intracellular signaling in these cells. We are particularly interested in phosphorylation of mucins, as they are known to be heavily glycosylated and have the ability to signal, and expression of the transcription factor T-bet, as B-cells deficient for T-bet did not effectively switch to IgG2a, IgG2b and IgG3 (Peng et al., 2002). Intracellular signaling of GALT leukocytes and intestinal
epithelial cells from perorally immunized C2GnT2-deficient mice and wild-type littermates will be determined. Muc1 phosphorylation will be assessed by Western blot, utilizing commercially available Muc1 and anti-phosphotyrosine antibodies (Quin and McGuckin, 2000). If necessary, mass spectrometry can also be utilized to determine Muc1 phosphorylation (Singh et al., 2007). Levels of T-bet expression will be determined by quantitative immunofluorescence of tissue sections using antibodies specific for T-bet and cell marker proteins (Johrens et al., 2006). Intracellular signaling of splenic leukocytes will also be determined as a control, as it is expected that the defect in C2GnT2-deficient mice that causes a reduction in the humoral response to mucosal antigens will be limited to the GALT.

h) Analyze O-glycan structures on intestinal epithelial cells and mucosal leukocytes that can serve as ligands for lectins, including galectins, by immunofluorescence and lectin blots.

The rationale for this aim is that glycan expression is regulated in leukocytes, and activation often induces glycan changes in leukocytes including DCs and lymphocytes. The functional significance of these glycan changes has been reported (Bax et al., 2007; Earl and Baum, 2008). Furthermore, glycans serve as ligands for various endogenous lectins, which also have altered expression following activation of leukocytes (Acosta-Rodriguez et al., 2004). Immunofluorescence will be performed on tissue
sections from perorally immunized C2GnT2-deficient and wild-type mice to compare the expression of glycan ligands for lectins, including galectin-1 and galectin-3. Tissue sections will be probed using commercially available, recombinant mammalian lectins. Conjugating these recombinant lectins to fluorescent tags will allow for easy visualization of the levels of expression of ligands for each lectin. This laboratory has experience using commercially available mammalian lectins to determine altered expression of endogenous ligands (Green et al., 2007). Using recombinant lectins will allow us to determine if any changes in glycan structures lead to an altered ability of these glycans to interact with immunologically relevant lectins, which would likely be functionally important. For additional glycan structure analysis we routinely collaborate with Dr. Anne Dell at Imperial College London for mass spectrometrical analysis of glycan structures (Wang et al., 2001; Chui et al., 2001). Dr. Dell has also collaborated with us in determining the O-glycan structures present in C2GnT2-deficient mice (Table 1.I; Table 2.I). In experiments for the primary research paper on these animals (Chapter 1) she was able to determine glycan structures of large O-glycans from the gastrointestinal tract including the colon using Matrix-assisted laser desorption/ionization-Time of flight Mass spectrometry (MS) and MS/MS techniques (Chapter 1). We will have the opportunity to collaborate with her, again, on this project if we find that we need more glycan structure information than lectins can provide.
While targeting glycosyltransferases in mammalian models has proven exceptionally insightful for the understanding of the numerous biomedical relevant functions glycans regulate (Grewal et al., 2008; Green et al., 2007; van Dyken et al., 2007; and Ellies et al., 1998), identification of the glycoprotein with altered glycan structure that causes the phenotype remains a challenge in the field of glycobiology. However, this laboratory has been successful in identifying the relevant glycoproteins and thus the mechanism for phenotypes seen in glycosyltransferase deficient mice (Ellies et al., 2002; Moody et al., 2003; Ohtsubo et al., 2005; Grewal et al., 2006). The approach this laboratory has taken that has proven successful is a four-step process. Step 1 - target the gene encoding the glycosyltransferase of interest in a mammalian model system, Step 2 - identify the phenotypes caused by deletion of that specific glycosyltransferase and the cellular basis of the given phenotype, Step 3 - determine glycoproteins involved, and Step 4 - determine the mechanism of the phenotype. Determining the actual glycoproteins involved is outside of the scope of this proposal due to time considerations. However, this proposal aims to further define step 2, which will includes further defining the phenotype in the absence of C2GnT2 and identifying the cell types involved in this phenotype, including the cell types with altered glycosylation. Furthermore data obtained based on this proposal should provide helpful in eventually identifying the relevant glycoproteins (step 3) and will allow us to begin to understand the mechanisms involved (Step 4).
i) Produce mice strains deficient in C2GnT2 specifically in hematopoietic or epithelial cell lineages, and determine the humoral response to KLH administered via the mucosal route and the mucosal barrier function in these mice.

To further define the cell type(s) that require C2GnT2 expression for an efficient humoral response to mucosal antigens it is necessary to generate mice in which C2GnT2 is deleted in specific cells types including epithelial cells and hematopoietic cells. To generate mice in which C2GnT2 is deleted from hematopoietic cells, male C2GnT2\(^{F/F}\) mice will be crossed to female mice expressing Cre-recombinase under the control of the Vav1 promoter (Vav-cre mice), which express Cre-recombinase in hematopoietic cells and the male germ line (Georgiades et al., 2002). Female offspring from this breeding will be bred to male C2GnT2\(^{F/+}\) to generate mice in which C2GnT2 is specifically deleted from hematopoietic cells and can be used in subsequent experiments. However, some expression of Cre-recombinase in the ovaries of Vav-cre mice has also been reported (de Boer et al., 2003), thus is may be necessary to use mice heterozygous for C2GnT2 in hematopoietic cells as control mice. An alternative would be to generate mice in which C2GnT2 is deleted specifically from B- and T-lymphocytes. To do this we would cross C2GnT2\(^{F/F}\) mice with hCD2-cre mice (de Boer et al., 2003). To generate mice in which C2GnT2 is deleted from epithelial cells C2GnT2\(^{F/F}\) mice will be crossed to Vil-cre mice, which express Cre-recombinase in epithelial cells (el Marjou et al., 2004).
Alternatively, bone marrow chimeras can be made. Mice in which C2GnT2 is deleted from specific cells types will then be immunized perorally with KLH and CT, and the development of antigen-specific systemic and mucosa humoral responses will be determined (Kjerrulf et al., 1997). The mucosal barrier function of these mice will also be assessed (Furuta et al., 2001), to determined if the decreased humoral response to mucosal antigens and increased mucosal permeability are linked in these mice.

Identification of the cell types involved in the phenotypes seen in the absence of C2GnT2 is essential for the eventual identification of the relevant glycoproteins involved and eventually to fully understand the mechanism leading to reduced humoral responses to mucosal antigens in the absence of C2GnT2 (see subaim 2h).

**Specific Aim 3: Investigate how loss of C2GnT2 modulates mucosal infections using *Citrobacter rodentium* as a model mucosal pathogen.**

*C. rodentium* is a murine model for infections with attaching and effacing pathogens, including human infections with enteropathogenic *Escherichia coli* (Mundy et al., 2005). Previous research has shown that mice lacking systemic humoral responses due to the loss of B-cells or CD4+ T-cells but not secreted IgA and IgM are susceptible to *C. rodentium* infection (Bry and Brenner, 2004; Masser et al., 2004). Additionally, literature suggests systemic IgGs, possibly including IgG2b, are necessary to effectively clear *C.
rodentium (Bry and Brenner, 2004, Masser et al., 2004; Bry et al., 2006). As systemic mucosal-antigen-specific IgG$_{2b}$ is reduced in C2GnT2-deficient mice, studying the humoral response to C. rodentium infection in C2GnT2-deficient mice compared to wild-types may help to define the specific IgG isotypes important for efficient clearance of C. rodentium. These experiments may also allow us to determine if the systemic humoral response required to effectively clear C. rodentium infection develops from mucosal presentation of the pathogen or possibly due to the pathogen breaching the mucosa and the systemic immune system gaining access to its antigens. Further knowledge of the role the systemic and mucosal immune system play in generating an efficient immune response to mucosal pathogens may lead to the development of more efficient oral vaccines and may help develop more efficient treatments for mucosal infections. Additionally, determining the role of glycans generated by C2GnT2 may play in regulating clearance of common infections may shed light on why this glycosyltransferase has been conserved in evolution.

a) Investigate the course of C. rodentium infection in C2GnT2-deficient mice and wild-type littermates including: survival, ability to clear the infection, and pathology caused by the pathogen.

C2GnT2-deficient mice will be perorally inoculated with $5 \times 10^7$ to $5 \times 10^9$ CFUs of C. rodentium. Following mucosal challenge with each
concentration of bacteria, mice will be monitored for survival for 48 days and moribund mice will be euthanized. Throughout the course of the experiment, fecal *C. rodentium* CFUs will be determined weekly to assess the ability of mice lacking C2GnT2 in specific cell types to clear the infection normally. All surviving mice will be euthanized on day 48 at which time CFUs in the colon, spleen, liver and blood will also be determined. Histological examination will be performed to assess colon damage and inflammation (Bry and Brenner, 2004; Maaser et al., 2004).

b) *Compare the systemic humoral response in C2GnT2-deficient and wild-type mice following mucosal infection with C. rodentium, to determine if the response is generated by the systemic or mucosal immune system.*

A concentration of *C. rodentium* that allows mice to survive for at least 21 days, as determined above, will be used to orally inoculate wild-type mice and C2GnT2-deficient mice. Sera and feces will be collected weekly from mice infected with this level of *C. rodentium*. Pathogen-specific immunoglobulin isotypes generated throughout the course of the infection will be determined by ELISA. Heat-killed *C. rodentium* will be immobilized, and fecal and sera samples will be probed with enzyme-conjugated antibodies specific for mouse immunoglobulin isotypes (Bry and Brenner, 2004).
Other Studies

The future directions discussed thus far in this section have focused on further understanding the role of C2GnT2 in mucosal immune function. However, this is not to suggest that my other research has not led to results worthy of additional studies. It would be interesting to further characterize the role of C2GnT2 in protection from colitis (Figure 1.4b-f) by outcrossing the C2GnT2-deficient mice to a mouse background more susceptible to colitis, such as 129/SvJ or C3H/HeJ Bir. Additionally, the increased mucosal permeability result in the C2GnT2-deficient mice (Figure 1.4a) should be followed up with a more physiological relevant mucosal barrier assay, for example by determining the ability of commensal or pathogenic bacteria to access the mesenteric lymph nodes.

Additionally, as C2GnT2 is highly expressed in mucin-producing and as Core 2 O-glycans interact with bacteria (Kawakubo et al., 2004; Ashida et al., 2008), we have initiated a collaboration with the Gordon Laboratory to investigate the microbiome of C2GnT2-deficient mice. My preliminary studies, using only 3 sets of littermates, did not reveal any substantial alterations in the composition of the microbiome in the absence of C2GnT2, when C2GnT2-deficient mice were co-housed with wild-type littermates (data not shown). The collaboration with the Gordon Laboratory will also reveal if loss of C2GnT2 in the mucosa causes alterations in commensal gene expression.
Furthermore, the remarkable induction of elongated O-mannose structures in mice deficient for all three C2GnTs (Table 1.II) is of interest, as the opposite O-glycosylation alteration (reduction in elongated O-mannose structures) causes some types of muscular dystrophy (Clement et al., 2008; Guglieri et al., 2008). Thus studies to investigate the expression of elongated O-mannose structures in brain and muscle tissue from mice deficient for all three C2GnTs may lead to increased understanding of the regulation of elongated O-mannose structures in these relevant tissues. Additionally, studies should be undertaken to determine if the increased elongated Core 1 O-glycan structures and elongated O-mannose structures in these mice (Table 1.II) are due to reduced competition for UDP-GlcNAc. UDP-GlcNAc concentrations can be increased by growing cells in media supplemented with GlcNAc (Sasai et al., 2002). The structures of O-glycans produced under these conditions could then be determined by MS. The results of such experiments may be greatly different based on cell types used. Alternatively, mice could be administered GlcNAc by gavage, and the intracellular concentration of UDP-GlcNAc could be determined as previously describes (Sasai et al., 2002). If the UDP-GlcNAc concentration was increased, O-glycan structures in the stomach could then be determined.
Acknowledgements

The future directions section is a modified version of the abstract, specific aims, and research designs and methods sections of a grant application entitled Glycosylation in the Control of Mucosal Immunity submitted to National Institute of Allergy and Infectious Diseases in response to a Request for Applications entitled Immune Defense Mechanisms at the Mucosa. I co-authored this grant application with Jamey D. Marth.
References


