Disruption of thymopoiesis in ST6Gal I-deficient mice

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Received on March 31, 2008; revised on May 18, 2008; accepted on May 28, 2008

Thymocyte development is accompanied by sequential changes in cell surface glycosylation. For example, medullary thymocytes have increased levels of α2,3-linked sialic acid and a loss of asialo core 1 O-glycans as compared to cortical thymocytes. Some of these changes have been linked to fine tuning of the T cell receptor avidity. We analyzed ST6Gal I transcript abundance and levels of α2,6-linked sialic acid across thymocyte subsets. We found that ST6Gal I transcript levels increased following T cell receptor β-selection suggesting that this sialyltransferase may influence the development of early thymocyte populations. Indeed, low levels of α2,6-linked sialic acid were found in the earliest T lineage cells, and then increased in T cell receptor β-selected cells. To determine whether ST6Gal I influences T cell development, we analyzed ST6Gal I deficient mice for disruptions in thymocyte populations. We found reduced thymic cellularity in the ST6Gal I deficient mice starting in the early thymocyte compartments.

Keywords: sialic acid/ST6Gal I/thymocyte development

Introduction

Sialic acids play multifaceted roles in intercellular processes within the immune system. Sialic acids are a family of 9-carbon sugars found at the nonreducing terminal position of glycoproteins and glycolipids. There is a distinct dichotomy in the roles that sialic acids play in the mammalian immune system. Sialic acid residues provide a mechanism by which toxins and pathogens, such as cholera toxin (Richards et al. 1979), influenza A (Suzuki et al. 2000), and enterovirus 70 (Nokhbeh et al. 2005), infiltrate host cells. Conversely, sialic acids regulate a diverse number of host immune responses (Varki 2007). For example, sialic acid moieties modulate the complement pathway via Factor H and inflammation pathways via the selectin family of sialic acid-binding proteins (Rosen 2004).

Sialic acid linkages are generated by a subset of glycosyltransferases, the sialyltransferases. Sialyltransferases catalyze the addition of sialic acid to glycoproteins and glycolipids during oligosaccharide production in the Golgi. Conclusive evidence highlighting the importance of sialylated moieties in the immune system has come from studies on mice that have targeted disruptions of specific sialyltransferases. ST3Gal IV-deficient mice have a partial deficiency in 2,3-linked sialic acid on Galβ1,4GlcNAc and as a result exhibit a loss of L-selectin-mediated leukocyte rolling (Sperandio 2006). ST3Gal I governs sialylation of the core 1 O-glycan Galβ1,3GlcNAc and loss of this gene product increases CD8 T cell apoptosis (Priatel et al. 2000). ST3Gal I−/− mice have further demonstrated that α2,3 sialic acid plays a protective function in regulating apoptosis in peripheral CD8+ T cells after clonal expansion by lymphocytic choriomeningitis virus or staphylococcal enterotoxin B infection (Van Dyken et al. 2007).

T cell maturation occurs in the thymus where thymocytes must complete several important developmental checkpoints or they die (Michie and Zuniga-Pflucker 2002). Monoclonal antibodies against CD4 and CD8 can broadly divide the developing thymocytes into four distinct populations. The earliest thymocytes are CD4− and CD8− (double negative, DN), whereas those cells that have successfully completed the early checkpoints express CD4 and CD8 and are referred to as double positive (DP) thymocytes. Finally, the DP cells transition to either single positive CD4+ or CD8+ cells prior to their exit to the periphery. The earliest DN populations can be further subdivided into four stages of development, DN1−4. Several of the key milestones in T cell development occur within the DN compartment, including the commitment to T cell lineage (DN2) and the first appearance of the TCR beta chain (β-selection) (DN3) (Dudley et al. 1994; Ceredig and Rolink 2002).

T cell development and maturation is accomplished by pronounced changes in sialylation. The α2,3 sialylation profile of thymocytes and mature T cells has been deduced from peanut agglutinin (PNA)-binding studies. PNA is a lectin that binds to Galβ1,3GlcNAc. The addition of an α2,3 sialic acid to this disaccharide abrogates PNA binding (Gillespie et al. 1992; Priatel et al. 2000). Select steps in T cell maturation and differentiation pathways are marked by changes in abundance of this α2,3-linked sialic acid. DN thymocytes bind PNA poorly (Holladay et al. 1993) indicating high levels of α2,3-linked sialic acid that subsequently decreases in the DP population found in the thymic cortex (Moody et al. 2001). Maturation to the single positive state and migration to the periphery correlate with an increase in α2,3-sialylation (Reisner et al. 1979; Moody et al. 2001). It is not until T cells become activated that there is again a selective loss of 2,3 sialylation (Chervenak and Cohen 1982; Hernandez et al. 2007). Memory T cells (CD8+CD44high) remain PNAhigh as they are hyposialylated for α2,3 sialic acid (Galvan et al. 1998).
Sialic acid in an α2,6 linkage to Galβ1,4GlcNAc has also been associated with immune regulation. This linkage is predominantly generated by the activity of one enzyme, ST6Gal I (Hennet et al. 1998; Martin et al. 2002). Targeted disruption of ST6Gal I demonstrated that ST6Gal I modulates inflammatory responses. Mice with this limited ST6Gal I defect present with abnormally elevated numbers of inflammatory cells after thioglycollate-induced peritonitis and an increase in the circulating pool of inflammatory cells due in part to an increased ability for granulopoietic generation (Nasirikenari et al. 2006). Mice deficient in ST6Gal I activity also show several B cell-related defects including low levels of serum IgM, a decrease in proliferation in response to IgM and CD40 crosslinking, reduced protein-tyrosine phosphorylation, and impaired antibody production in response to both T-dependent and T-independent antigens (Hennet et al. 1998). Functionally, the B cell defect has been linked in part to the role that α2,6-linked sialic acid plays in modulating B cell activation via the lectin, CD22 (Hennet et al. 1998; Ghosh et al. 2006).

While a humoral defect was evident in the ST6Gal I-deficient mice (Hennet et al. 1998), no severe defect in T cell development has been reported to date. There is evidence, however, that 2,6-linked sialic acids influence T cell development and differentiation. For example, the addition of sialic acid in an α2,6-linkage to CD45 inhibits galectin-1-induced apoptosis in medullary thymocytes (Amano et al. 2003). Recently, the lectin SNA (Martin et al. 2002), SNA binding was used to characterize a significant loss of α2,6-linked sialic acid in anti-CD3-activated T cells compared with naive T cells. This activation-related loss correlated with a decrease in ST6Gal I transcripts (Comelli et al. 2006).

Recently, we utilized Affymetrix microarrays to investigate the transcript changes in pre- and post-β-selected thymocytes from wild-type mice. One of the transcripts which increased following β-selection was ST6Gal I. Furthermore, lectin staining showed an increase in α2,6-linked sialic acid in the post-β-selected DN4 thymocytes. These results prompted us to further evaluate the potential role of the α2,6 sialyltransferase in T cell development. Postulating that sialic acid might play a yet undiscovered role in β-selection, we obtained ST6Gal I-deficient mice (Hennet et al. 1998) in order to assess T cell development in the absence of 2,6-linked sialic acid. We found a disruption in thymocyte development in the ST6Gal I-deficient mice, characterized by decreased numbers of thymocytes starting in the earliest DN subpopulation and changes in several apoptosis-related RNA transcripts.

Results

ST6Gal I transcript abundance varies across thymocyte development

Microarray data from six different thymocyte subsets were probes to determine the transcript abundance of ST6Gal I (Figure 1) across thymocyte development. The data represented in the figures are from five replicate chips of five separate sorts of each population. Surface expression levels of CD25, CD44, TCR, CD69, CD4, and CD8 were also analyzed and all correlated with the microarray data (data not shown). As shown in Figure 1, ST6Gal I transcript abundance increases following β-selection (the transition from DN3 to DN4).

![Fig. 1. Changes in transcript abundance profiles for ST6Gal I across six discrete thymocyte populations.](https://academic.oup.com/glycob/article-abstract/18/9/719/1988073)

Siaα2,6Gal levels differ between populations of early thymocytes

Since the expression of the ST6Gal I product (Siaα2,6Gal) can be assessed using the lectin SNA (Martin et al. 2002), SNA binding was used to determine if ST6Gal I transcript abundance was reflective of the Siaα2,6 glyctype of the DN3 and DN4 populations. The level of SNA staining on the DN1, DN2, DN3, and DN4 thymocytes is shown in Figure 2. There is a loss of Siaα2,6Gal moiety in DN2 and the DN3 populations. The subsequent increase in SNA binding of thymocytes in the DN4 population correlates with the increase in ST6Gal I transcript abundance described above (Figure 1).

ST6Gal I deficiency impairs thymocyte development

The transient increase of α2,6-linked sialic acid in the DN4 population led us to evaluate ST6Gal I-deficient mice to determine if ST6Gal I played a role in thymocyte development. Figure 3 shows the difference in SNA staining between wild-type and ST6Gal I-deficient mice across the four major thymocyte subsets. We assessed the numbers of total thymocytes and the main thymocyte subpopulations in wild-type and ST6Gal I−/− mice at 3, 8, and 12 weeks of age. Since no T cell defect has previously been reported for the ST6Gal I−/− mice we were surprised to find that overall numbers in the thymus were decreased in these mice (Figure 4).

Next, we sought to determine if the decrease in thymic cellularity was attributable to the loss of a single thymocyte subpopu-lation or was ubiquitous, encompassing all subpopulations of the ST6Gal I−/− thymus. Figures 5 and 6 show comparatively the breakdown of the major thymocyte populations between wild-type and ST6Gal I−/− mice. In Figure 5, cellularity in the earliest thymocytes (DN1 cells) was analyzed by gating to exclude cells positive for CD4, CD8, or non-T lineage markers as described in Materials and methods. The remaining cells were broken down into four DN subpopulations based on CD25, cKit, and CD44 expression of each population. Surface expression levels of CD25, CD44, TCR, CD69, CD4, and CD8 were also analyzed and all correlated with the microarray data (data not shown). As shown in Figure 1, ST6Gal I transcript abundance increases following β-selection (the transition from DN3 to DN4).
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Fig. 2. The percentage of Siaα2,6Gal expressing cells is decreased in the DN2 and DN3 thymocyte compartments. Total thymocytes were harvested from C57BL/6 female mice 6–8 weeks of age. Cells were stained as described in Materials and methods. Populations were defined as DN1 (Lin−/CD4−/CD8−/TCRγδ−/CD44+/CD25−), DN2 (Lin−/CD4−/CD8−/TCRγδ−/CD44+/CD25+), DN3 (Lin−/CD4−/CD8−/TCRγδ−/CD44−/CD25+), and DN4 (Lin−/CD4−/CD8−/TCRγδ−/CD44−/CD25−). (A) Representative histogram of SNA binding in the double negative compartment of C57BL/6 mice (open) (n = 5). Filled histogram is representative of background staining in the absence of SNA. (B) Percentage of gated cells from each DN population that is positive for SNA staining. Error bars represent standard deviations of the mean (n = 3). (C) Average median fluorescent intensity of SNA staining in the DN compartment. Error bars represent standard deviations of the mean (n = 3).

<0.0001, 0.0063, and 0.0013, respectively. The DN3 population (CD25+CD44−) from the ST6Gal I−/− was significantly decreased in the 8-week-old mice (P = 0.0222) and at 12 weeks of age (P = 0.0120). Likewise, the DN4 cells (CD25−CD44−) were decreased to a significant extent in the ST6Gal I-deficient mice at the 8 week (P = 0.0129) and again at the 12-week time point (P = 0.0108).

The loss of cellularity in the ST6Gal I-deficient mice was also observed in the more mature DP (CD4+CD8+), SP4 (CD4+CD8−), and SP8 (CD4−CD8+) thymocyte populations

Fig. 3. SNA staining in the four main thymocyte populations. Total thymocytes were harvested from either C57BL/6 female mice or ST6Gal I−/− mice at 8 weeks of age. Cells were stained as described in Materials and methods. Populations were defined as DN (CD4−/CD8−), DP (CD4+/CD8+), SP4 (CD4+/CD8−), and SP8 (CD4−CD8+). (A) Representative histogram of SNA staining of ST6Gal I−/− mice (filled) or C57BL/6 (dotted line). Bold line represents a no SNA control on a C57BL/6 mouse. (B) Percentage of gated cells from each wild-type population that was positive for SNA staining. Error bars represent standard deviations of the mean (n = 3). (C) Average median fluorescent intensity of SNA staining in the wild-type mouse. Error bars represent standard deviations of the mean (n = 3).
Materials and methods

Thymic cellularity is significantly decreased in ST6Gal I-deficient mice at 8 and 12 weeks of age. Thymuses were harvested from C57BL/6 (closed bars) or ST6Gal I<sup>−/−</sup> (open bars) female mice at 3 weeks, 8 weeks, or 12 weeks of age. Bars represent the mean number of cells from at least seven mice (the number of mice used for the analysis of each population is detailed in Materials and methods). Error bars represent standard deviation between the individual mice. P values are indicated as <0.0005<sup>***</sup>.

(Figure 6). A statistically significant difference was not observed at 3 weeks of age; however, at 8 and 12 weeks of age the DP populations were significantly decreased (P = 0.0004 and P < 0.0001, respectively). The same pattern was observed in the SP populations with significant decreases in cellularity seen in the ST6Gal I-deficient mice at 8 and 12 weeks of age in both the SP4 and SP8 populations.

Markers for B cells (CD19), NK cells (NK1.1), erythroid cells (Ter119), macrophages (Mac-1), and myeloid cells (Gr1) were also examined (data not shown). No significant differences in the numbers of these cell types were observed in the thymuses of ST6Gal I-deficient mice compared to age-matched wild-type mice, indicating that the loss of thymic cellularity in the ST6Gal I-deficient mice is restricted to cells of the T lineage.

Early thymocytes from ST6Gal I-deficient mice have increased transcription of several genes involved in apoptosis

Microarray analysis comparing early thymocyte populations between ST6Gal I-deficient mice and their wild-type counterparts suggests that the loss of thymic cellularity could be attributable to increased apoptosis (Figure 7). Transcripts for apoptosis-regulating genes, annexin A2 (Huang et al. 2008), programmed cell death 1 (CD279), granzyme B (Waterhouse et al. 2006), tumor necrosis family receptor subfamily 9, killer cell lectin-like receptor subfamily B member 1A, OX-2 (Voisin et al. 2006), and BCL2A1 (Rasooly et al. 2005) were all increased in the ST6Gal I-deficient mice at 7 weeks of age in cKit<sup>+</sup>CD25<sup>−</sup>−DN thymocytes (Figure 7A). It is plausible that the loss of early DN populations due to increased apoptosis results in reduced numbers of thymocytes in the subsequent compartments. We also observed differences between wild-type and ST6Gal I-deficient mice in transcript levels for several other genes, such as CD96, in the DN2 and DN3 populations (Figure 7B–C). To demonstrate the validity of the data from the gene chips, relative transcript abundance for several differentially expressed genes was assessed via RT-PCR. Levels of transcripts for CD96, ENTPD4 (ectonucleoside triphosphate disphosphohydrolase), and CAP1 (adenyl cyclase-associated protein) correlated with the changes indicated on the microarrays (data not shown).

Discussion

Because significant changes in sialylation are known to accompany cancer (Dall’Olio 2000; Hedlund et al. 2008) and autoimmune disease (Carsons 2002), it is important to understand the functional role that sialic acid plays in lymphocyte development so that novel therapeutic strategies to combat these pathologies may be developed. The precise role that ST6Gal I plays in thymocyte development is currently unknown; however, our data
complement of T lineage precursors.

suggest that the lack of this enzyme leads to a loss of thymocytes beginning at the DN1 stage of thymocyte development (Figure 5). This loss could be attributable to either a block in thymocyte development, inefficient seeding of the thymus, or differences in levels of apoptosis. The DN2 stage is where T cell lineage commitment is thought to occur (Moore and Zlotnik 1995; Zuniga-Pflucker et al. 1995; Schmitt et al. 2004; Masuda et al. 2007). We looked at various lineage markers to assess whether the loss of ST6Gal I shunted nascent thymocytes into non-T cell developmental pathways but did not see significant changes in these populations in the ST6Gal I-deficient thymuses (data not shown). Additionally, we did not see a compensatory shift or an increase in the numbers of any single T lineage population that might suggest a developmental defect or block in the T lineage. Experiments in our lab (unpublished data) and others (Hennet et al. 1998) suggest that peripheral mature T cell functionality as judged by anti-CD3 stimulation remains intact in ST6Gal I-null mice as in their wild-type counterparts. It has been shown that ST6Gal I-null mice have an increased number of myeloid cells in the bone marrow (Nasirikenari et al. 2006). It is possible that this defect partially explains the loss of thymocytes as the bone marrow may be at capacity, overcrowded or inhospitable, to the full complement of T lineage precursors.

We utilized Affymetrix gene ontology annotations to mine the microarray data for changes in well-characterized cellular pathways. This analysis revealed that the DN1 (CD25–cKit+ ) population (Figure 7A) in the ST6Gal I-deficient mice had increased levels of transcript abundance for genes that enhance sensitivity to apoptosis, such as annexin A2 (Huang et al. 2008) population (Figure 5 which could potentially allow for a small contaminat-

![Fig. 6. There is a significant loss of DP and SP thymocytes in ST6Gal I−/− mice. Thymocytes were from either C57BL/6 (closed bars) or ST6Gal I−/− (open bars) female mice of the indicated ages. Double positive and single positive populations were defined as follows: DP (CD4+CD8+), SP4 (CD4+CD8−), and SP8 (CD4−CD8+). Each bar represents the average number of cells from at least seven mice (the number of mice used to generate each data point is described in Materials and methods) and error bars represent the standard deviation between the individual mice. P values are indicated as <0.05*, <0.005**, <0.0005***.

![Fig. 7. There is a significant increase in the transcript abundance of several apoptosis-related genes in ST6Gal I−/− mice. (A) Transcript levels of genes from multiple apoptotic pathways from either C57BL/6 (closed bars) or ST6Gal I−/− (open bars) female mice at 7 weeks of age. Early DN thymocytes (cKit−CD25+) were isolated as described in Materials and methods. (B) Differences in transcript abundance in several genes between wild-type (closed bars) and ST6Gal I−/− (open bars) DN2 (CD25+CD44+) thymocytes. (C) Differences in transcript abundance in several genes between wild-type (closed bars) and ST6Gal I−/− (open bars) DN3 (CD25+CD44+) thymocytes. The data shown are derived from five replicate chips of five separate sorts of each population. Error bars represent the standard error of the mean. Abbreviations used in this figure include: TRAT1, T cell receptor-associated transmembrane adaptor 1; CaMKIIb, calmodulin-dependent protein kinase II beta, ENTPD4, ectonucleoside triphosphate diphosphohydrolase-4.

The data provided evidence that there are potentially important differences in the apoptosis pathways of thymocytes from ST6Gal I mice compared to those from wild-type mice. The microarray
data also revealed an intriguing loss in the expression of CD96 transcript in the ST6Gal I-deficient DN2 and DN3 thymocytes when compared to their wild-type counterparts. CD96 is the receptor molecule for CD155 and nectin-1 (Seth et al. 2007). CD96 plays a putative role in cellular adhesion which may point to a thymocyte migration disturbance in the absence of ST6Gal I.

While it has been largely accepted that α2,6-linked sialic acid is only present on mature medullary thymocytes (Baum et al. 1996), this study shows that in the mouse it is expressed transiently in several of the DN populations. Previously the SNA binding to the DN1 and DN4 populations would have been difficult, if not impossible to observe, unless the thymocytes had been parsed into these small subsets. It remains to be elucidated as to which glycoprotein is binding SNA in these early populations. It is possible that apoptosis in the early thymocyte populations is regulated by lectins other than galectin-1.

The glycosylation of proteins and lipids in the Golgi is governed in part by a competition between the various glycosyltransferases. For example, ST6Gal I-deficient mice show increased levels of fucosylated Galβ1,4GlcNAc residues (Martin et al. 2002). This may indicate that the observed changes in the thymic cellularity of ST6Gal I−/− mice are not as severe as might be expected due to partial compensation by the addition of other competing sugars on the Galβ1,4GlcNAc stalk.

Shifting in sialic acid residues on T cells occur during normal development in the thymus, activation in the periphery, and can even be indicative of disease states (reviewed in Ohtsubo and Marth 2006). As shown, variations in ST6Gal I expression occur early in T cell development (Figure 1). Likewise, it has previously been demonstrated that ST6Gal I expression varies in even the most mature peripheral T cell subsets as STGal I transcript is downregulated in antigen-specific CD8 T cells after viral infection as compared to naive CD8 T cells (Kaech et al. 2002). This study furthers our understanding of the changes in glycosylation that occur during normal murine thymocyte development. It is evident from these data that ST6Gal I plays an important role in murine thymopoiesis.

Materials and methods

Mice

C57BL/6 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Harlan Sprague Dawley (Indianapolis, IN). ST6Gal I-deficient mice were obtained through the consortium of Functional Glycomics. Mice were housed and bred in a U.S.D.A. approved facility at the University of Tulsa in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council). Animal protocols were approved by the Institutional Animal Care and Use Committees of the University of Tulsa and the University of Oklahoma Health Sciences Center.

Thymocyte isolation

Single cell suspensions were generated as previously described (van De Wiele et al. 2004; Marino et al. 2006). Briefly, tissues were passed through 70 μm nylon screens using 3 mL syringe plungers and complete tumor media (CTM) (Teague et al. 2000). Cell suspensions were treated with a RBC lysis buffer (Sigma-Aldrich, St. Louis, MO), washed into CTM, and enumerated using a hemocytometer or a Coulter Z2 Particle Counter and Size Analyzer (Beckman Coulter, Fullerton, CA).

Microarray comparison of thymocyte subpopulations in normal mice

Thymocytes from 4- to 5-week-old C57BL/6NHsd mice were sorted into six defined populations: DN3, DN4, DP TCRαβ, DP TCRγδ, SP4 CD69−, and SP8 CD69+. For the DN3 and DN4 sort purifications, mAbs (purchased from BD Pharmingen) against the following antigens were used to eliminate DPs and other lineage (Lin) cells: CD4, CD8, TCRγδ, CD19, Mac-1, NK1.1, Ter-119, and Ly-6G&C (van de Wiele et al. 2007). The CD4- and CD8-specific mAbs were directly coupled to APC whereas the other mAbs were biotinylated, and streptavidin-APC was used as a secondary reagent. Cells were further discriminated into DN3 and DN4 using anti-CD44-FITC and anti-CD25-PE mAbs. Lin−/CD4−/CD8−/CD44−/CD25− cells were sorted to obtain the DN3 population and Lin−/CD4−/CD8−/CD44−/CD25− cells were sorted to obtain the DN4 population. Cells DP for CD4 and CD8 were further sorted into TCR negative and TCR intermediate populations using biotinylated mAb against TCRβ (H57-597) followed by streptavidin-PerCP. Cells single positive for either CD4 or CD8 were sorted using an additional marker CD69 (H1.2F3) in order to discriminate less mature (CD69+/−) and more mature (CD69+) single positive cells. Total RNA was isolated from 150,000 cells of each population using Qiagen Mini-Elute columns. The RNA was then amplified with two cycles of cDNA synthesis and in vitro transcribed according to the GeneChip Eukaryotic Small Sample Labeling Assay Version II protocol (Affymetrix, Santa Clara, CA). Labeled RNA was hybridized to the gene chips (Mouse Genome 430A) and the University of Tulsa Microarray Core Facility was utilized for chip processing and statistical analysis. Data were collected and analyzed with Affymetrix Microarray Suite (MAS, V5.0), dChip (V1.3), and Spot Fire DecisionSite (7.3, V11.0). The data represented in the figures are from five replicate chips of five separate sorts of each population.

Lectin staining

In Figure 2, DPs, SPs, and other non-T lineage (Lin) cells were eliminated as described above, with the exception that streptavidin-PerCP rather than streptavidin-APC was used as a secondary reagent. Cells were further discriminated into...
DN1 through DN4 populations using anti-CD44-APC and anti-CD25-PE mAbs. In Figure 3, thymocyte subsets were distinguished using CD4-APC and CD8-PE. In both figures, the detection of Siaα2,6Gal was assessed by binding to FITC-coupled SNA (Sambucus nigra) (Vector Laboratories, Burlingame, CA).

**Phenotyping stains**

Single cell suspensions of thymocytes and peripheral lymphocytes were incubated with purified CD16/CD32 (Fc Block) and subsequently stained with the monoclonal antibodies indicated in the figure legends. In Figures 4–6, the total number of mice analyzed for each data point was as follows: 3-week WT (n = 8), 3-week ST6Gal I−/− (n = 8), 8-week WT (n = 10), 8-week ST6Gal I−/− (n = 9), 12-week WT (n = 9), and 12-week ST6Gal I−/− (n = 7).

**Microarray comparison of DN populations between wild-type and ST6Gal I−/− mice**

Thymocytes from 7-week-old wild-type C57BL/6Nhsd mice and ST6Gal I-deficient mice were sorted into three populations: DN1, DN2, and DN3. Biotinylated antibodies were used to eliminate DPs, SPs, and other lineage (Lin) cells as described above except streptavidin-PE used as a secondary reagent. Cells were further discriminated into DN1, DN2, and DN3 using anti-cKit-APC and anti-CD25-FITC mAbs. LIN−/CD4−/CD8−/cKit+/CD25− cells were sorted to obtain the DN1 population, LIN−/CD4−/CD8−/cKit+/CD25+ cells to obtain the DN2 population, and LIN−/CD4−/CD8−/cKit−/CD25+ cells to obtain the DN3 population. Total RNA was isolated from 10,000 cells of each population using Qiagen Mini-Elute columns. The RNA was then amplified with two cycles of cDNA synthesis and in vitro transcribed according to the Eukaryotic Target Preparation protocol (Affymetrix, Santa Clara, CA). Labeled RNA was hybridized to the gene chips (Mouse Genome 430A 2.0) and the University of Tulsa Microarray Core Facility was utilized for chip processing and statistical analysis. Data were collected and analyzed with Affymetrix GeneChip Operating Software (GCOS version 1.1.1), dChip(V1.3), and SAS (version 9.1.3 for Windows/PCs). The data represented in the figures are from five replicate chips of five separate sorts of each population.

**Funding**

This work was supported by grants from the National Institutes of Health/Oklahoma BRIN (P20RR16478), the Mervin Bovard Foundation, and the Oklahoma Center for the Advancement of Science and Technology (HR03-124). This work was also supported by funds from the University of Oklahoma College of Medicine Department of Surgery; kindly provided by the department chair Dr. Thomas A. Broughan.

**Acknowledgements**

We thank Debbie Neal for secretarial support and Chunxiao Fu for microarray preparation work.

**Conflict of interest statement**

The authors are not aware of any conflicts of interest.

**Abbreviations**

APC, aliphocytocyanin; CTM, complete tumor media; DN, double negative; DP, double positive; Fc, constant fragment; FITC, fluorescein isothiocyanate; Gal, galactose; GlcNac, N-acetyl-D-glucosamine; MALDI-TOF, matrix assisted laser desorption ionization time of flight; NK, natural killer; PE, phycoerythrin; PerCP, peridinin chlorophyll; PNA, peanut agglutinin; SNA, Sambucus nigra agglutinin; SP, single positive; ST, sialyltransferase; TCR, T cell receptor.

**References**


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