Sialyltransferase mRNA abundances in B cells are strictly controlled, correlated with cognate lectin binding, and differentially responsive to immune signaling in vitro

Julie H. Marino, Matt Hoffman, Megan Meyer, and Kenton S. Miller

Faculty of Biological Science and The Mervin Bovaird Center for Studies in Molecular Biology and Biotechnology, 600 S. College Ave., University of Tulsa, Tulsa, OK 74104

Received on April 7, 2004; revised on July 6, 2004; accepted on July 26, 2004

Mouse gene knockout studies have provided unimpeachable evidence of immune-relevant functions for several sialyltransferase enzymes including ST6Gal I, ST3Gal I, and ST3Gal IV. Such studies cannot, however, identify cellular mechanisms for regulating such activities. In this article we provide evidence that murine B lymphocytes respond to specific immune signals in vitro with tightly regulated changes in the sialic acid composition of the cell surface glycocalyx. These changes are both quantitative and qualitative in nature and are apparently regulated at both the transcriptional and posttranscriptional levels. We used lectin binding and flow cytometry combined with relative real-time PCR to show that MAH and PNA binding are tightly correlated with the abundance of ST3Gal IV and ST3Gal I mRNA, respectively, under several different conditions of B cell stimulation. Finally, we show that although SNA binding and the expression of ST6Gal I coding sequence are not tightly correlated, there is a clear differential control of 5′UTR exon usage in response to different immune signals.

Key words: B lymphocyte/glycocalyx/lectin/sialyltransferase/Q-RT PCR

Introduction

The past decade has witnessed numerous discoveries defining new roles for sialylation in both the innate and adaptive immune responses. For example, both the siglec (Crocker and Varki, 2001) and selectin (Ellies et al., 2002b; Lowe, 2002) families of immune cell receptors require linkage-specific sialylated glycans for receptor binding. On the other hand, binding of the galectin family of receptors is blocked by glycan sialylation (Rabinovich et al., 2002). Furthermore, sialylation of the glycans carried on many well-characterized immune receptors is now known to modulate the binding affinity and/or specificity of such receptors, including CD8 (Moody et al., 2003), CD22 (Collins et al., 2002), CD43 (Priatel et al., 2000), CD44 (Katoh et al., 1995), CD45 (Yu et al., 2002), and CD169 (Barnes et al., 1999), as well as immune adhesion mediating integrins such as β1 (Semel et al., 2002).

Over 20 different enzymes are currently known to mediate such sialylations, and they constitute a family of transferases that fall into three distinct subfamilies based on their general substrate and linkage specificities (Tsujii et al., 1996). There seems to be significant substrate overlap between sialyltransferases (STases) within a subfamily, at least when assayed in vitro (Tsujii, 1996). However, Marth and co-workers have constructed several informative sialyltransferase gene knockout mice (KO), including the ST6Gal I−/− (Hennet et al., 1998), ST3Gal I−/− (Priatel et al., 2000), and ST3Gal IV−/− (Ellies et al., 2002) lines. These studies demonstrate that each transferase has at least one unique and immunologically important substrate phenotype.

Although the ST6Gal I gene is expressed in multiple tissues throughout the body including the nervous system (Dall’Olio, 2001), the only major defect reported in the ST6Gal I KO mouse is an impaired humoral immune response. This is evidenced by reduced levels of circulating immunoglobulin M (IgM), impaired B cell proliferation in response to various activation signals, and impaired antibody production in response to both T-dependent and T-independent antigens. Both surface IgM and CD22 levels are significantly reduced on ST6Gal I KO B cells as well (Hennet et al., 1998). Sialic acid in an α2,6-linkage is at least part of the preferred ligand for CD22, a known regulator of B cell receptor (BCR) signaling (Tedder et al., 1997); however, the ST6Gal I KO has a more severe phenotype than a CD22 KO, suggesting that loss of 2,6-linked sialic acid has other effects on the immune system beyond functioning as a ligand for CD22 (Hennet et al., 1998). Again, although the ST3Gal I gene is widely expressed, the only immediately apparent defect in the KO mouse is an almost total absence of peripheral CD8+ T cells, which are lost by apoptosis, perhaps induced via CD43 clustering (Priatel et al., 2000). It has been recently suggested that ST3Gal I–mediated sialylation of CD8β modulates its affinity for major histocompatibility locus (MHC) I during thymocyte maturation (Moody et al., 2003). Collectively these studies provide solid evidence that the sialylation state of the immune cell glycocalyx is critical for the proper immune function and that changes to the sialylation state are under direct developmental control. Yet to be identified are the specific immune and genetic mechanisms controlling such changes.

Plant lectins have proven to be invaluable tools for the structural analysis of both the immune cell glycocalyx and its constituent glycoproteins and glycolipids, and for
characterizing the general sialylation state of various tissues in both normal and KO mice (Martin et al., 2003). We use flow cytometry to quantify binding of the sialic acid-specific lectins from Sambucus nigra (SNA) and Maackia amurensis (MAH) and the GaLβ1,3GalNAc-specific lectin Arachis hypogaea (PNA) to murine splenic B cells following in vitro activation. We show that any of several well-defined immune signals induce significant and differential changes in the lectin-binding phenotype of the activated cells. Furthermore, using real-time relative reverse transcriptase polymerase chain reaction (RT-PCR), we show that for two of the three STases considered here such changes are well correlated with the mRNA abundance of the STase putatively responsible for synthesizing the lectin binding site. Finally, we show that the ST6Gal I gene exhibits differential exon usage in its 5' untranslated region (UTR) in response to differential signaling through various BCRs.

Results

In vitro B cell activation

In vivo, B lymphocyte-stimulating antigens fall into two broad categories: those that are dependent on T cell help (T-dependent) and those that are not, which are of two types (T-independent type I and type II). For the in vitro activation of purified splenic B cells, we have used anti-CD40 monoclonal antibody-mediated cross-linking of cell surface CD40 to model T-dependent activation and goat anti-mouse IgM F(ab')2-mediated cross-linking of membrane-bound IgM to model T-independent type II activation (DeFranco, 1999).

As shown in Figure 1, in vitro cross-linking of sIgM or CD40 led to the rapid appearance of CD69, a well-characterized marker of lymphocyte activation (Sanchez-Mateos and Sanchez-Madrid, 1991). Expression

![Fig. 1. Signaling through cell-surface IgM or CD40 but not the IL-2 or IL-4 receptor activates small resting B cells. Purified B cells were activated by incubation MLC medium supplemented with reagents as indicated in each panel. After 42 h, the cells were harvested and assayed by flow cytometry for expression of CD69 following incubation with a phycoerythrin-conjugated anti-CD69 monoclonal antibody (thick line) compared to resting cells stained with the same antibody (thin line).](image-url)
of intercellular adhesion molecule-1 (CD54) and CD44 were also increased (data not shown). Anti-CD40 but not anti-IgM stimulation also leads to increased CD80 expression, whereas incubation in culture medium alone is sufficient for significant CD86 expression (Bagriacik and Miller, 1999). When used alone, neither interleukin (IL)-2 nor IL-4 (Figure 1) nor any other tested cytokine (IL-6 and interferon [IFN]-γ), affected these particular markers, nor did the cells proliferate significantly in response to any of these reagents administered individually (data not shown). However, incubation in IL-4 alone did enhance expression of MHC class II molecules, and treatment with IL-4, IL-6, or IFN-γ led to increased B cell survival in culture as assessed by propidium iodide staining (data not shown). From these studies we conclude that the B cell preparations and reagents are behaving in a manner consistent with the known responses of small resting B cells to the specific stimulatory signals tested (DeFranco, 1999).

**MAH binding and ST3Gal IV expression are strongly correlated**

MAH recognizes sialic acid linked α(2,3) to Galβ1,4GlcNAc, Galβ1,3GlcNAc and Galβ1,3GalNAc. Thus changes in MAH binding could be mediated by changes in the activity of either ST3Gal I or II (preference for Galβ1,3GlcNAc), ST3Gal III (preference for Galβ1,3GlcNAc), or ST3Gal IV or VI (preference for Galβ1,4GlcNAc); increased sulfation of nonreducing terminal lactosamines may also contribute to MAH binding. As shown in Figure 2, MAH binding is only significantly elevated following anti-IgM or anti-CD40 stimulation and is actually reduced somewhat following incubation in IL-2 alone.

The initial hypothesis for these experiments was that there might be a strong correlation between changes in the expression level of a glycan on the B cell surface as revealed by lectin binding and changes in the expression level of the mRNA encoding the glycosyltransferase responsible for its synthesis. Such a possibility has been suggested for at least some STase genes (Gillespie et al., 1992; Ruan et al., 1999). To test this hypothesis, we used relative real-time RT-PCR to quantify the relative amounts of the relevant STases mRNAs (Marino et al., 2003). As shown in Table I, all mRNAs tested responded with statistically significant differential alterations in gene expression ratio in response to all treatment conditions tested. The 95% confidence intervals for each measurement is show in parentheses.
RNA was isolated from B cells incubated for 42 h under various conditions as noted and real-time relative RT-PCR was performed as described in Materials and methods. The number shown is the natural log of the mRNA expression ratio relative to resting B cells and the 95% confidence intervals for this determination are noted in parentheses (Marino et al., 2003).

### Table I. Relative mRNA abundances versus treatment conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ST6Gal I</th>
<th>Core 2</th>
<th>ST3Gal I</th>
<th>ST3Gal III</th>
<th>ST3Gal IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>1.8 (1.7, 2.0)</td>
<td>-0.7 (-0.6, -0.8)</td>
<td>-0.4 (-0.3, -0.5)</td>
<td>1.1 (1.0, 1.2)</td>
<td>-1.3 (-1.1, -1.3)</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.0 (1.9, 2.1)</td>
<td>-0.7 (-0.6, -0.7)</td>
<td>0.0 (-0.1, 0.1)</td>
<td>0.8 (0.7, 0.9)</td>
<td>0.5 (0.4, 0.6)</td>
</tr>
<tr>
<td>a-IgM</td>
<td>1.0 (0.9, 1.1)</td>
<td>-1.1 (-1.0, -1.2)</td>
<td>-1.3 (-1.2, -1.6)</td>
<td>0.0 (-0.2, 0.1)</td>
<td>1.4 (1.2, 1.5)</td>
</tr>
<tr>
<td>a-IgM +IL-4</td>
<td>1.8 (1.8, 1.9)</td>
<td>-0.7 (-0.6, -0.7)</td>
<td>-0.7 (-0.6, -0.7)</td>
<td>0.7 (0.6, 0.8)</td>
<td>1.0 (0.9, 1.1)</td>
</tr>
<tr>
<td>a-CD40</td>
<td>0.8 (0.8, 0.9)</td>
<td>-0.4 (-0.3, -0.4)</td>
<td>-0.1 (-0.1, -0.2)</td>
<td>0.2 (0.1, 0.3)</td>
<td>0.2 (0.0, 0.3)</td>
</tr>
<tr>
<td>a-CD40 + IL-4</td>
<td>3.2 (3.0, 3.3)</td>
<td>-0.2 (-0.2, -0.3)</td>
<td>-0.7 (-0.6, -0.7)</td>
<td>1.4 (1.2, 1.5)</td>
<td>1.5 (1.4, 1.7)</td>
</tr>
</tbody>
</table>

### Table II. Correlation: lectin binding versus STase expression

<table>
<thead>
<tr>
<th>Lectin</th>
<th>ST6Gal I</th>
<th>Core 2</th>
<th>ST3Gal I</th>
<th>ST3Gal III</th>
<th>ST3Gal IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA</td>
<td>-0.1</td>
<td>-0.5</td>
<td>-0.9**</td>
<td>-0.2</td>
<td>0.8'</td>
</tr>
<tr>
<td>SNA</td>
<td>0.5</td>
<td>-0.4</td>
<td>-0.5</td>
<td>0.4</td>
<td>0.8'</td>
</tr>
<tr>
<td>MAH</td>
<td>0.0</td>
<td>-0.3</td>
<td>-0.5</td>
<td>0.2</td>
<td>0.9***</td>
</tr>
</tbody>
</table>

A paired value Student’s t-test was conducted comparing values for the relative expression of the various STase mRNAs with the binding of the various lectins for B cells incubated under the conditions listed in the table. Correlation coefficients (r) and one-tailed p-values for estimation of significance are reported. Where a p-value is not reported, the correlation was not statistically significant. 

*p < 0.05, **p < 0.01, ***p < 0.001.

**PNA binding and ST3Gal I expression are also strongly correlated**

The enzyme ST3Gal I preferentially sialylates the O-linked glycan core 1 substrate Galβ1,3GalNAc on the nonreducing galactose. Unfortunately, there is no known lectin that uniquely recognizes this sialylation pattern. However, PNA binds most strongly to nonsialylated, unbranched, O-linked core 1 structures, thus any loss of sialylation should lead to increased PNA binding. PNA binding is known to be a marker of germinal center B cells in mice, thus its mode of regulation in B cells is of some interest (Lahvis and Cerny, 1997). Of all reagents tested singly, only anti-IgM F(ab')2 was able to cause significant up-regulation of PNA receptors (Figure 4); however, other reagents used in combination could also lead to increased PNA binding. These included anti-CD40 plus IL-4 (Figure 4), and lipopolysaccharide plus IL-4 (data not shown). On the other hand, loss of PNA binding compared to resting cells was not observed for any condition of incubation.

The expression ST3Gal I mRNA and the binding of PNA are very significantly but inversely correlated (r = -0.9, p < 0.01), but there was no statistically significant correlation between PNA binding and core 2 mRNA abundance (r = -0.5, p > 0.1). Thus, although core 2 enzyme can
compete with ST3Gal I for core 1 structures and thus lead to increased PNA expression, this does not seem to be the case here. The expression of ST3Gal II mRNA, whose enzyme sialylates Galβ1,3GalNAc on glycolipids, was either unchanged or increased under all conditions of incubation and was not significantly correlated with the binding of any of the lectins examined (data not shown). In other experiments we have shown that the neuraminidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid is without effect on the expression of PNA binding glycans under any conditions of B cell incubation (Miller, unpublished data). Thus, we conclude that the increased PNA binding seen following some conditions of incubation is most likely regulated by the observed changes in ST3Gal I mRNA expression.

SNA binding and ST6Gal I expression are poorly correlated

SNA prefers sialic acid linked α2,6 to lactosamine but may bind to 2,6-linked sialic acid on other structures as well (Brinkman-Van der Linden et al., 2002). Compared to freshly isolated resting B cells, SNA binding was significantly increased under all conditions of incubation (Figure 5), including incubation in medium alone (data not shown). It has been shown that SNA binding is virtually abolished in ST6Gal I KO mice (Martin et al., 2003), but despite compelling evidence that ST6Gal I is the sole enzyme responsible for the presence of Sia-α2,6-Gal on the lymphocyte cell surface (Hennet et al., 1998), there is no significant correlation between SNA binding and the expression of ST6Gal I coding sequence (r = −0.5, p > 0.1). This lack of correlation could result from one or more of several reasons. First, there may be a limited number of substrate molecules on which ST6Gal I can act, and any increase in enzyme activity above some threshold level saturates these substrates. It is also likely that there are posttranscriptional mechanisms controlling enzyme activity as well and that such mechanisms are also differentially affected by the various incubation conditions. Finally, it is possible that undefined membrane changes could lead to differential accessibility of SNA for binding sites, thus uncoupling the quantitative relationship between ST6Gal I expression and SNA binding.

Fig. 4. PNA binding is significantly increased following anti-IgM stimulation. Purified B cells were activated by incubation MLC medium supplemented with reagents as indicated in each panel. After 42 h, the cells were harvested and assayed by flow cytometry for the binding of phycoerythrin-conjugated PNA (thick line) compared to resting cells stained with the same reagent (thin line).
ST6Gal I 5'UTR sequences are differentially expressed following differential B cell stimulation

Because it had been shown that there are multiple promoters capable of initiating transcription of the ST6Gal I coding sequence in murine B cells (Wuenesch et al., 2000), we decided to assay the relative expression of the various ST6Gal I 5'UTR sequences associated with these promoters. As shown in Figure 6, the abundance of each 5'UTR changes independently in response to the various stimuli; however, correlation analysis revealed no significant relationship between expression of any one 5'UTR exon and SNA binding to the cell surface.

On the other hand, the expression of coding sequence and the expression of the X2 5'UTR were very well correlated \((r = 0.97, p < 0.001)\). Therefore it is likely that the X2 5'UTR is making the greatest contribution to the occurrence of the coding sequence in most incubation conditions. The H and X3 5'UTRs of the ST6Gal I gene were also assayed, but neither was detectable at 24 h. However, X3 was detectable at 72 h in anti-CD40+IL-2+IL-4

---

**Fig. 5.** SNA binding is increased under all conditions of B cell incubation. Purified B cells were activated by incubation MLC medium supplemented with reagents as indicated in each panel. After 42 h, the cells were harvested and assayed by flow cytometry for the binding of phycoerythrin-conjugated SNA (thick line) compared to resting cells stained with the same reagent (thin line).

**Fig. 6.** ST6Gal I 5'UTR exon abundance is controlled via differential receptor signaling. Purified splenic B cells were incubated for 24 h under the conditions indicated. ST6Gal I coding sequence and 5'UTR exon usage was quantified by relative real-time RT-PCR. Error bars represent 95% confidence intervals.
stimulated cultures, that is, in plasma cells (data not shown). It has been reported that the P1 promoter, which gives rise to exon H bearing mRNAs, is responsive to IL-6 stimulation of hepatocytes (Dalziel et al., 1999). Incubation of resting B cells with IL-6, although stimulating an increase in the abundance of Q-O, X1, and X2 exons, did not lead to the presence of detectable H exons; however, H exon-containing transcripts were easily detected in control RNA from mouse hepatocytes (data not shown).

Because all ST6Gal I 5’UTRs seem to show enhanced relative expression when IL-4 was in the culture medium (Figure 6) and because anti-IgM induced signaling seemed to suppress ST3Gal I expression (Table I), we grouped the data with respect to the IL-4 and anti-IgM treatment conditions and performed a Student’s t-test. Of the three ST6Gal I UTR sequences assayed, only X1 exhibited statistically different abundances between incubation conditions that either did or did not contain IL-4 (p < 0.001). The enhanced expression of X1 in the presence of IL-4 is not simply a result of an enhanced viability of these cultures because the anti-CD40+IL-2 cultures are just as viable at all time points as assessed by propidium iodide staining (data not shown). Thus IL-4 seems to be the primary stimulus for the increased abundance of X1 under any of the conditions tested.

Discussion

Mouse gene KO studies have been instrumental in defining immune-relevant roles for several glycosyltransferases (Ellies et al., 2002; Henne et al., 1998; Priatel et al., 2000). Such studies, however, cannot address the signaling mechanisms by which such genes are regulated nor identify the requisite signals themselves. In this study we have begun to identify and to dissect such mechanisms for three sialyltransferase genes of immune interest.

ST3Gal IV

MAH is known to recognize 2,3-linked sialic acid bound either to Galβ1,4(3)GlcNAc-R or Galβ1,3GalNAc-R. In some instances it may also recognize 3-linked sulfate bound to these same substrates (Martin et al., 2002). Despite its apparent broad specificity, in this study we find that the correlation between MAH binding and ST3Gal IV mRNA abundance is extremely significant. Furthermore, examining the individual conditions of incubation reveals that MAH binding is significantly increased following mIgM cross-linking (Figure 2) and, of all ST3Gal transferases assayed (I–VI), only ST3Gal IV mRNA abundance is specifically increased following such treatment (Table I and unpublished data). Anti-IgM treatment also leads to strong expression of PNA binding, suggesting that increased 2,3 sialylation of glycans other than Galβ1,3GalNAc-R is responsible for the increased MAH binding and that ST3Gal IV does not significantly compete with ST3Gal I for the Galβ1,3 GalNAc-R substrate in these cells. This observation is consistent with studies of ST3Gal IV−/− mice in which PNA binding to lymphocytes was found to be only modestly increased (Ellies et al., 2002).

The study of Ellies and colleagues also found that of the four ST3Gal KOs tested (I–IV), ST3Gal IV contributed most significantly to selectin ligand formation (sLex) as measured by P and E selectin binding. In studies from our laboratory sLex expression as measured by monoclonal antibody binding was increased under all incubation conditions tested relative to resting cells (Marino, unpublished data), including incubation in media plus IL-2 alone, a condition in which ST3Gal IV expression is significantly decreased (Table I). However, Ellies et al. noted that the reduction in E/P selectin binding to neutrophils seen with ST3Gal IV KO mice relative to wild type was significantly less than that seen with core 2 KO mice, thus implicating at least one other sialyltransferase in sLex biosynthesis in neutrophils. It is probable that this other enzyme (probably ST3Gal VI) is also at least partially responsible for significant sLex synthesis in B cells, and we find that ST3Gal VI mRNA abundance is indeed significantly increased (1.4-fold) following B cell incubation in IL-2 (Marino, unpublished data). This also suggests that sLex represents only a small fraction of all possible MAH binding sites in B cells and that most such sites are synthesized by ST3Gal IV.

Interestingly, the strong correlation between MAH binding and ST3Gal IV mRNA abundance also suggests that enzyme activity is tightly controlled by mRNA abundance and that there is little if any posttranscriptional control exerted on this enzyme under any of our assay conditions. This tight correlation of mRNA abundance and enzyme activity is also consistent with the observation of haploinsufficiency for this gene (Ellies et al., 2002). Surprisingly, ST3Gal IV expression was also significantly correlated with SNA and PNA binding (Table II); however, this is most likely reflective of the strong correlations between MAH and PNA binding (0.9, p < 0.001) and between MAH and SNA binding (0.9, p < 0.001), which is probably indicative of the tight control maintained over the structure of the B cell glycoalyx in response to various differentiation signals.

ST3Gal I

Increased PNA binding to core 1 structures (Galβ1,3GalNAc-seryl/hr) could result from changes any one of three enzyme activities: (1) a reduction of ST3Gal I leading to exposure of core 1 structures, (2) an increase in the activity of the core 1 enzyme (β1,3-galactosyltransferase) in excess of the ability of ST3Gal I to modify the core 1 structure, or (3) an increase in core 2 enzyme, which competes with ST3Gal I for the core 1 substrate and whose activity does not block PNA binding. In most tissues, including the spleen, PNA binding is only modestly increased in the ST3Gal I KO mouse; however, binding to kidney glomeruli, the adenal cortex, and the thymic medula is significantly increased (Martin et al., 2002), suggesting that blocking of terminal Galβ1,3GalNAc is mediated by solely ST3Gal I activity in these tissues, whereas in most other tissues additional STase activities may also participate. Binding of tomato lectin, which recognizes linear polylactosamine attached to core 1 structures, is significantly increased in the spleen of the ST3Gal I KO mouse, presumably due to increases in core 2 branched structures created by core 2 enzyme in the absence of terminal sialylation by ST3Gal I (Martin et al., 2002); however, tomato lectin binding is not increased in anti-IgM-activated B cells (data not shown),
presumably due to reduced core 2 enzyme activity resulting from reduced core 2 mRNA expression (Table I). Furthermore, it is known that CD45 is the principal carrier of PNA binding sites on murine B cells following activation with LPS/dextran and IL-2 (Cook et al., 1987) and that core 2 enzyme is required for the synthesis of the B220 epitope on CD45 (Ellies et al., 2002). In our hands, PNA binding is increased and B220 binding and core 2 mRNA abundance are significantly reduced following LPS stimulation of murine B cells (Marino et al., unpublished data), which is consistent with the results of Cook et al. (1987). Interestingly, however, B cells activation via anti-IgM is not accompanied by a decrease in B220 binding, even though there is a significant reduction in core 2 mRNA expression. Furthermore, the principal PNA binding protein seen in western blots of cell surface proteins from anti-IgM activated cells is not CD45 (Marino et al., unpublished data), suggesting that the mechanisms for the appearance of PNA receptors under the two conditions of activation may be different. Further experiments are required to resolve this issue.

**ST6Gal I**

Gene KO studies have established that ST6Gal I is the enzyme responsible for generating most if not all SNA binding sites in the adult mouse (Martin et al., 2002) and although a second ST6Gal transferase (ST6Gal II) has been identified in humans (Krzewinski-Recchi et al., 2003; Takashima et al., 2002), this enzyme seems to make no significant contribution to 2,6-linked sialic acid on lymphocytes. It was surprising therefore that ST6Gal I coding sequence abundance and SNA binding were not significantly correlated in this study. Possible explanations for this observation are: (1) substrates for the ST6Gal I enzyme are saturated following even the relatively modest increases in mRNA expression (2.2-fold) seen following anti-CD40 stimulation, thus uncoupling SNA binding and ST6Gal I mRNA expression, (2) the posttranslational control of ST6Gal I enzyme activity by phosphorylation (Breen and Georgopoulos, 2003; Gu et al., 1995), and/or (3) the differential translation of ST6Gal I mRNAs containing different 5'UTRs. With respect to this last possibility, analysis of the 5'UTR sequences with the MFOLD 3.1 RNA secondary structure program (Zuker et al., 1999) suggests that the 5'UTRs have very different structures with very different folding energies, Q-O being the most stable at -170 to -210 kcal, whereas X2 at -35 to -40 kcal and X1 at -75 to -90 kcal are significantly less stable. These numbers are also consistent with correlations between 5'UTR abundance and SNA expression, with Q-O being negatively correlated and X1 and X2 positively correlated. However, these correlations were not statistically significant, and further studies are needed to validate this conclusion.

Studies from other laboratories have also shown that immune signaling molecules can modulate ST6Gal I expression. Hanasaki et al. (1994) demonstrated that both tumor necrosis factor α and IL-1 could mediate induction of ST6Gal I coding sequences in human endothelial cells and caused a differential sialylation of several cell surface proteins; in 1999, Lau’s laboratory (Dalziel et al., 1999) demonstrated that incubation in IL-6 caused increased expression from the H exon–encoding PI promoter active in mouse hepatocytes, implicating it in the acute phase response. However, subsequent studies using PI gene KO mice failed to confirm this suggestion (Appenheimer et al., 2003). In this study we demonstrate that IL-4 signaling can control the mRNA abundance levels of X1-containing ST6Gal I transcripts.

### Materials and methods

#### Mice

Six- to 8-week-old C57BL/6, originally obtained from Jackson Laboratories were bred and maintained in our local animal facility and were used for all experiments.

#### Preparation of resting small B cells

B cells were prepared as previously described (Bagriacik and Miller, 1999). Briefly, spleen cells are treated with NH$_4$Cl/Tris, pH 7.2, to lyse red blood cells and then incubated in MLC (RPMI-1640 supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol, 5 μM HEPES, 10% fetal bovine serum) for 1 h at 37°C, 5% CO$_2$ to remove adherent cells. Nonadherent cells are separated on a five-layer Percoll. These cells are treated with a combination of anti-Thy1.2, anti-CD4, anti-CD8, anti-Mac1α, anti-CD86, and anti-CD80 monoclonal antibodies at 4°C for 30 min and are depleted by low-tox guinea pig complement lysis (Accurate Chemical, Westbury, NY) for 45 min at 37°C. Depleted cells were separated on a five-layer Percoll (Pharmacia, Uppsala, Sweden) gradient (50%, 60%, 70%, 80%, and 100%) and cells from 60–70% interface are recovered.

#### Cell activation

B cells were activated by incubation in 24-well tissue culture plates in complete medium (10%/ml) containing either anti-CD40 (1 μg/ml) or anti-IgM (30 μg/ml) with or without IL-4 (5 ng/ml) or IL-2 (30 U/ml). After 24–48 h, depending on the experiment, cells were harvested and used for lectin staining or RNA isolation.

#### Cell staining with lectins and antibodies and flow cytometry

Cell staining with lectins was performed as previously described (Bagriacik and Miller, 1999). All cells were stained with biotinylated lectins from Vector Labs (Burlingame, CA). Five × 10$^6$ cells were stained in 100 μl final volume of HBSS containing 5–20 μg/ml lectin for 30 min in the dark at 4°C. The cells were then counterstained with phycoerythrin-conjugated streptavidin (PharMingen, San Diego, CA) and fixed with 2% formaldehyde prepared in Hank’s balanced salt solution (HBSS). For antibody staining, cells (10$^6$/ml) were first incubated with an Fc receptor blocker (anti-CD16/CD32) according to the manufacturer’s recommendations, and then titrated concentrations of monoclonal antibodies were conjugated either with phycoerythrin or biotin in 100 μl HBSS containing 0.01% sodium azide for 30 min at 4°C. The cells were then washed and fixed in 2% formaldehyde prepared in

1272
HBSS. For biotin-labeled antibody staining, phycoerythrin-conjugated streptavidin (Pharmingen, San Diego, CA) was used as a secondary reagent. Cells stained with lectins or antibodies were analyzed using an EPICS 751 flow cytometer interfaced with a Cicero data acquisition unit running Cyclcop software (Cytomation, Fort Collins, CO).

RNA was prepared from cell suspensions using TRIzol Reagent (Gibco BRL Gaithersburg, MD) according to the manufacturer’s instructions. Quality and quantity of RNA was assessed by measuring the A260/A280 ratio and by analysis on ethidium bromide–stained 1% agar gels with and without heating to 65°C for 10 min in sterile, diethyl pyrocarbonate–treated, 18 MΩ water. Gels were imaged on a Molecular Dynamics Storm and analyzed using ImageQuant software (Amersham Pharmacia Biotech, Piscataway, NJ). Any RNA preparations exhibiting significant qualitative or quantitative differences between heated and unheated samples were rejected. Isolated RNA was treated with DNase I (Invitrogen, Carlsbad, CA) prior to use for cDNA synthesis.

cDNA synthesis and PCR amplification

Reverse transcription was performed using 5 μg total RNA, random primers, and SuperScript II RT (Invitrogen) in a total volume of 20 μl. The reaction was incubated at 25°C for 10 min, followed by incubation at 42°C for 50 min. cDNA synthesis was followed by RNase H treatment (Roche Molecular Biochemicals, Indianapolis, IN). To minimize potential effects of differential synthesis during the RT reaction, three separate cDNA reactions were pooled for each RNA preparation analyzed. Real-time PCR is carried out using a Smart Cycler thermal cycler (Cepheid, Sunnyvale, CA). Each PCR reaction included 2.5 μl of 10× PCR buffer without MgCl2 (Sigma-Aldrich, St. Louis, MO), 1.0 μl 25 mM MgCl2, 0.5 μl 10 mM dNTPs (Perkin-Elmer, Wellesley, MA), 20 pM each primer, 0.25 μl Taq DNA Polymerase (Sigma-Aldrich), 0.2 μl 30% bovine serum albumin (Sigma-Aldrich), SYBR Green I (Molecular Probes, Eugene, OR) at a final concentration of 1:20,000 dilution of the commercial stock and an appropriate volume of the cDNA preparation. PCR cycling conditions included a 94°C heating step for 1 min at the beginning of every run. The tubes were then cycled at 94°C for 30 s, annealed at 62–68°C for 0.5 min, and extended at 72°C for 30 s. Optical data were collected during the annealing step. A melting curve was generated at the end of every run to ensure product uniformity.

The primers used for Q-RT PCR were: 18S rRNA forward: 5’-CCAAGACAGAATCGGAGGT-3’; 18S rRNA reverse: 5’-GGACATCTAAAGGGCATCAG-3’; ST3Gal I forward: 5’-GGAGGAGACATACCCGGTG-3’; ST3Gal I reverse: 5’-GGAGTCTTCCAGGTTCGG-3’; ST3Gal III forward: 5’-TGAGGATCCACCCGCTATTTCCG-3’; ST3Gal III reverse: 5’-GCCCTCACTGTAGCCGGAGCTTAT-3’; ST6Gal I coding forward: 5’-GTCCTGAAGAGAAGCGGAC-3’; ST6Gal I coding reverse: 5’-TCACAGATTCTCCTCGGTGA-3’. Each primer pair has been validated by sequencing of the resultant RT-PCR product. All primers were designed using the X-Primer program and synthesized by the Recombinant DNA/Protein Resource Facility at Oklahoma State University (Stillwater, OK).

Data analysis

Optical data were exported from the Cepheid Smart Cycler as comma-separated values files (+.csv) and imported into MS Excel. We wrote a Visual Basic Excel macro that facilitates determination and conversion of the appropriate Smart Cycler optics data to a logarithmic format for subsequent analysis. The resultant data is then pasted into SAS JMPIN 4.0 for determination of slopes, intercepts, and their respective standard errors and correlation coefficients, which are subsequently pasted into a second spreadsheet for final calculation of relative expression level (Marino et al., 2003). Correlation analysis and Student’s t-tests were conducted using Prism 3.0 (GraphPad Software, San Diego, CA).

Acknowledgments

This work was supported by grants to K.S.M. from the National Institute of Allergy and Infectious Diseases (AI 41164), The Mervin Bovaird Center for Molecular Biology and Biotechnology, and the University of Tulsa Office of Research.

Abbreviations

BCR, B cell receptor; HBSS, Hank’s balanced salt solution; IFN, interferon; IL, interleukin; Ig, immunoglobin; KO, knockout; MAH, Maackia amurensis agglutinin II; MHC, major histocompatibility locus; PNA, Arachis hypogaea agglutinin; RT-PCR, reverse transcriptase polymerase chain reaction; SNA, Sambucus nigra agglutinin; STase, sialyltransferase; UTR, untranslated region.

References


