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Activation of Murine CD4+ and CD8+ T Lymphocytes Leads to Dramatic Remodeling of N-Linked Glycans1

Elena M. Comelli,2* Mark Sutton-Smith,‡ Qi Yan,* Margarida Amado,* Maria Panico,* Tim Gilmartin,† Thomas Whisenant,‡ Caroline M. Lanigan,† Steven R. Head,† David Goldberg,§ Howard R. Morris,3‡ Anne Dell,‡ and James C. Paulson4*  

Differentiation and activation of lymphocytes are documented to result in changes in glycosylation associated with biologically important consequences. In this report, we have systematically examined global changes in N-linked glycosylation following activation of murine CD4 T cells, CD8 T cells, and B cells by MALDI-TOF mass spectrometry profiling, and investigated the molecular basis for those changes by assessing alterations in the expression of glycan transferase genes. Surprisingly, the major change observed in activated CD4 and CD8 T cells was a dramatic reduction of sialylated biantennary N-glycans carrying the terminal NeuGc2-6Gal sequence, and a corresponding increase in glycans carrying the Galα1-3Gal sequence. This change was accounted for by a decrease in the expression of the sialyltransferase ST6Gal I, and an increase in the expression of the galactosyltransferase, α1-3GalT. Conversely, in B cells no change in terminal sialylation of N-linked glycans was evident, and the expression of the same two sialyltransferases was increased and decreased, respectively. The results have implications for differential recognition of activated and unactivated T cells by dendritic cells and B cells expressing glycan-binding proteins that recognize terminal sequences of N-linked glycans. The Journal of Immunology, 2006, 177: 2431–2440.

D ifferentiation and activation of lymphocytes are accompanied by programmed remodeling of cell surface glycans of glycoproteins with biologically important consequences. Notably, the conversion of the peanut agglutinin (PNA)5 high (PNAhigh) phenotype of immature CD4/CD8 medulatory thymocytes to the PNAlow phenotype of the mature single-positive CD8 and CD4 thymocytes results from conversion of the PNA ligand, the O-linked glycan Galβ1-3GalNAcThr/Ser, to its sialylated form, NeuAca2-3Galβ1-3GalNAcThr/Ser, that is no longer recognized by PNA due to increased expression of a sialyltransferase, ST3Gal I (1–4). In CD8 T cells, this glycosylation change reduces the affinity of CD8 for MHC class I, suggesting that sialylation of CD8 O-glycans modulates CD8 function during selection and maturation of CD8 T cells (5–7). Naïve CD8 cells of ST3Gal I−/− mice that are constitutively PNAhigh undergo rapid apoptosis in the periphery, reducing the CD8 population to 10% of wild type (2). Yet, conversion from PNAlow to PNAhigh is a natural consequence of activation of wild-type CD8 cells resulting from down-regulation of ST3Gal I (2, 8, 9). In contrast, ST3Gal I is differentially regulated in activated and polarized Th1 and Th2 CD4 cells leading to the PNAhigh and PNAlow phenotype, respectively (10). The additional increased expression of fucosyl- and sialyltransferases in Th1 cells promotes synthesis of the selectin ligand sialyl-Lewis X (NeuAca2-3Galβ1-4[Fucα1-3]GlcNAc), while Th2 helper cells lack sialyl-Lewis X sequences because a key fucosyltransferase, Fuc-T VII, is not expressed. Such differential glycosylation accounts for the selectin-mediated recruitment of CD4 Th1 cells to sites of inflammation (11–17).

Insights into the importance of N-linked glycan structures in lymphocyte biology have also been obtained by ablation or overexpression of key glycosyltransferases. For example, ablation of the GlcNAc transferase Mga5 leads to increased TCR signaling and autoimmune disease, and promotes Th2 over Th1 responses, a result of the loss of N-linked glycans with a GlcNacβ1-6Man branch that interacts with galectins and reduces TCR signaling by restricting TCR clustering (18). A sialyltransferase that elaborates the terminal sequence NeuAca2-6Galβ1-4GlcNAc on N-linked and O-linked glycans has been shown to block the binding of galectin-1 that induces T cell death by clustering of CD45 and reduction of its phosphatase activity (19). The same sequence has been documented to be the glycan ligand for CD22 (Siglec-2), a regulator of B cell signaling, that has been proposed to mediate adhesion of B cells to T cells (20–25). The expression of the enzyme responsible for the synthesis of the sequence, ST6Gal I, was noted to be down-regulated in a microarray analysis of Ag-activated CD8 cells, but the consequences on the structures of CD8 cell glycans were not investigated (26).

Despite the importance of glycosylation in lymphocyte function, changes in glycosylation are largely studied through indirect means such as probing for the presence or absence of specific
glycan sequences with plant or animal lectins or carbohydrate-specific Abs. Although this approach has provided biologically important information, it is limited to examining aspects of structure relevant to the specificity of the probes used, and does not address the extent to which changes occur. Indeed, there have been few investigations of glycosylation changes in lymphocytes in which direct analysis of glycan structure has been conducted. An exception is a rigorous analysis conducted nearly 20 years ago investigating the O-linked glycans of human leukosialin before and after activation of human T cells (27). This study revealed that the predominant disialylated “core 1” glycans of naive T cells (NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcThr/Ser) were replaced by larger branched “core 2” structures (NeuAcα2-3Galβ1-3(NeuAcα2-6Galβ1-4GlCNacβ1-6)GalNAcThr/Ser) in activated cells (27), now known to be the nonfucosylated precursor of the O-linked sialyl-Lewis x ligand of P-selectin (28). The change was concluded to result from altered expression of two glycosyltransferases that form the NeuAcα2-6GalNAc linkage and GlcNAcβ1-6GalNAc linkages, enzymes that compete to form the alternative branch points in these structures, respectively.

In principle, such analysis can now be conducted by high throughput methods for assessment of glycan structure and expression of the glycosyltransferase genes responsible for their synthesis (29). To this end, we have investigated changes in glycosylation of N-linked glycans of resting and activated murine T and B lymphocytes using MALDI-TOF mass spectrometry (MS) profiling for assessment of glycan structure, and a custom gene microarray based on Affymetrix technology, developed and annotated by the Consortium for Functional Glycomics (www.functionalglycomics.org)) for evaluating changes in expression of glycosyltransferase genes. A dramatic change was observed in the terminal glycosylation pattern of N-linked glycans of CD4 and CD8 T cells. In freshly isolated cells, the predominant complex type N-glycans contained terminal sialic acid, in the NeuGcα2-6Gal sequence that forms the ligand of murine CD22 and is implicated in regulation of galectin-mediated cell death. In contrast, the activated cells exhibited a dramatic decrease in sialylated glycans, and a corresponding increase in glycans with the Galα1-3Gal terminal sequence. Gene microarray analysis revealed that the changes in N-glycan structures in activated T cells corresponded to changes in the expression of a sialyltransferase and galactosyltransferase responsible for the synthesis of these terminal structures. The approach of using MALDI-TOF MS for profiling glycan structure coupled with gene microarray analysis to survey glycosyltransferase expression may prove to be a powerful combination for investigating glycosylation changes in differentiating or activated cells.

Materials and Methods
Preparation and culture of splenocytes
C57BL/6 male mice (6–10 wk old) were obtained from The Scripps Research Institute Custom Breeding core and used to prepare splenocytes, as previously described (9). In this report, fresh cells refer to cells not subjected to cell culture, and resting and activated cells are those subjected to cell culture in the absence or presence of activating agents, respectively. For T cell activation experiments, splenocytes were plated (10⁶ cells/plate) with immobilized anti-CD3 (BD Pharmingen) and cultured for 72 h in RPMI 1640 medium supplemented with either 4 ng/ml IL-2 and 5 ng/ml IL-12, or 4 ng/ml IL-2 and 10 ng/ml IL-4, or 4 ng/ml IL-2 (R&D Systems). For B cell activation, splenocytes were cultured for 72 h in RPMI 1640 medium supplemented with 10 μg/ml anti-IgM (Jackson ImmunoResearch Laboratories) and 10 ng/ml IL-4.

Lymphocyte purification
CD4 and CD8 T cells and B cells were purified from either fresh, resting, or activated splenocytes using the MidiMACS system (Miltenyi Biotec) by positive selection, according to the manufacturer’s protocols. Briefly, cells were resuspended in PBS containing 5 mg/ml BSA and 2 mM EDTA and incubated with anti-CD4, anti-CD8, or anti-CD19 microbeads for 15 min at 4°C for purification of CD4, CD8 T cells, or B cells, respectively. After washing, cells were resuspended in the same buffer and applied to the column. Purity of cell preparations was always ≥90%, as judged by flow cytometry. Purified cells were either washed with PBS twice and immediately frozen and stored at −80°C until use or immediately used for flow cytometry analysis.

Flow cytometry
Purified CD4, CD8 T cells and B cells were aliquoted in aliquots of 5 × 10⁵ cells in 100 μl of PBS containing 10 mg/ml BSA with or without GS-I-B-FITC lectin from Griffonia simplicifolia (GS; EY Laboratories) at a concentration of 20 ng/ml or Sambucus nigra agglutinin (SNA) lectin (Vector Laboratories) at a concentration of 40 μg/ml, for 30 min on ice. Flow cytometry data were acquired on viability-gated cells using a FACSCalibur flow cytometer and analyzed with the CellQuest software system (BD Biosciences).

Analysis of glycan transferase gene expression
Analysis of gene expression was conducted using a custom gene microarray (GLYCOv1 chip) produced by Affymetrix for the Consortium for Functional Glycomics (www.functionalglycomics.org), and containing probe sets for 122 glycosyl- and sulfotransferases (29). Three independent experiments were performed for each activation condition of each lymphocyte type (CD4, CD8, and B lymphocytes), resulting in triplicate RNA samples per condition. RNA was extracted using the Qiagen RNeasy mini kit according to the manufacturer’s protocol. After treatment with DNase I amplification grade (Invitrogen Life Technologies), total RNA was purified with RNeasy columns using the cleanup protocol (Qiagen) and was quality checked with an Agilent Bioanalyzer (Agilent Technologies). For glycosyltransferase expression analysis, 5 μg of total RNA was amplified and biotin-labeled using the Bioarray High Yield RNA Transcript Labeling kit (Enzo Life Sciences) and then hybridized to the GLYCOv1 chip. One chip per biological replicate was run. Hybridization and scanning of the chip were performed according to the Affymetrix recommended protocols.

The MAS 5.0 algorithm (Affymetrix) was used to determine present (P) and absent (A) absolute calls for each sample. All marginal (M) calls were interpreted as absent. Genes were finally considered present if they had been assigned a present call in at least two of the three biological replicate samples (Group Call). Expression signals were then generated using the Robust Multiarray Analysis algorithm (Ref. 30 and http://www.berkeley.edu/~bolstad/RMAExpress/RMAExpress.html). Unsupervised hierarchical clustering by sample was performed with BRB ArrayTools using the default settings for correlation and linkage (http://linus.nci.nih.gov/BRB-ArrayTools.html).

Statistical comparisons were made for each gene to determine whether the signals in the fresh and activated samples were statistically significant. Accordingly, the robust multiarray analysis (RMA) signals of the triplicate fresh samples of each lymphocyte type were compared with the signals of the triplicate samples of each activation condition corresponding to that lymphocyte type. The CD4 and CD8 T cell experiments, which involved three different activation conditions, warranted the use of the Dunnnett’s test, a modified t test, which controls for the “experiment wise” or “per experiment” error rate (31, 32). It does not produce a p value, rather the computed q value is compared against a table of Dunnnett’s critical values determined for the appropriate α value as follows:

\[ q' = t_{\text{dunn}} = \frac{x_i - \bar{x}_i}{\sqrt{\frac{2 \text{MSE}}{n}}} \]

where: MSE = \[ \frac{\sum_{i=1}^{k} x_{i}^2 - \left(\frac{\sum_{i=1}^{k} x_{i}}{n-1}\right)^2}{k} \]

\[ k \] is the number of groups, and \( n \) is the sample size.

A two-tailed Dunnnett’s test was applied. The critical values for α = 0.05, \( k = 4 \) groups, degrees of freedom (error) = \( v_e = (n - k) = 12 - 4 = 8 \), were:

\[ q' = t_{\text{dunn}}(2) = q(\sqrt{\frac{k}{n-k}}) = q(0.05, 8, 4) = 2.88 \]

For the B cell experiments, the two groups were fresh and activated, and a Student t test, assuming the variances were equal, was applied (the result of an F max test for variance on all probe sets was that the variance was...
equal for all but three cases, and in all three cases the variance was very small, with the control sample variance effectively 0):

\[ t_{\text{critical}}(2) = t\left(\frac{N}{2}\right) = t\left(\frac{10}{\sqrt{2}}\right) = 2.776 \]  

(3)

Characterization of B and T lymphocytes N-glycans

Approximately 25 million fresh and 15 million activated B and T lymphocytes, respectively, were used for each analysis. Cell preparations were homogenized, detergent extracted, reduced/carboxy methylated, dialyzed, and digested with trypsin as previously described (33). N-glycans were released from each proteolyzed extract by peptide/N-glycanase (PNGase F; Roche) treatment and were subsequently permethylated using the sodium hydroxide procedure, as described elsewhere (33). MALDI-TOF MS, electrospray MS/MS, and methylation analyses were then performed following previously published strategies (34).

Results

Strategy for analysis of changes in N-linked glycosylation

The overall strategy for analysis of changes in N-linked glycosylation was to activate freshly isolated mixed splenocytes in vitro for 72 h, purify the cell type of interest, and either subject the purified cells to glycan analysis, or extract mRNA for analysis on the glycoprotein microarray. Each preparation of lymphocytes involved pooling splenocytes from 10 to 30 mice, and subjecting them to activation conditions selected for T cell activation (anti-CD3 with IL-2/IL-12 or IL-2/IL-4) or B cell activation (anti-IgM with IL-2/IL-4) as described in Materials and Methods. The activation conditions for T cells were chosen to determine whether early glycosylation changes were influenced by cytokines that promote CD4+ T cell polarization to Th1 (IL-2/IL-12) and Th2 (IL-2/IL-4) subsets. Lymphocytes were purified by positive selection using either anti-CD4-, anti-CD8-, or anti-CD19-coated magnetic microbeads for CD4 T cells, CD8 T cells, or B cells, respectively, yielding cell populations 90–98% pure (Fig. 1). The remaining cells were discarded. Activated cells are compared with fresh lymphocytes because lymphocytes cultured in the absence of cytokines were subject to extensive cell death. Three independent experiments were done for each activation condition for mRNA extraction and microarray analysis, and at least two experiments for each activation condition were subjected for evaluation of N-glycan structure by MALDI-TOF MS analysis.

Analysis of N-glycans from fresh and activated lymphocytes

Portions of representative MALDI-TOF MS profiles showing the mass region with complex N-glycans are shown in Fig. 2 for fresh and activated T and B lymphocytes. Striking differences are observed upon comparison of fresh and activated CD8 and CD4 T cells as illustrated by the major peaks annotated in symbol format. The predominant peaks in the fresh cells are disialylated biantennary and monosialylated triantennary glycans. In contrast, the predominant peaks in the cells activated with IL-2/IL-12 are di- and tribranched structures disubstituted with dihexose termini. Similar
profiles were observed for CD8 and CD4 T cells activated with IL-2/IL-4 (data not shown). As an example of the dramatic change between fresh and activated cells, note that the predominant peak representing the disialylated biantennary glycan at mass 3027 in fresh CD8 and CD4 cells is largely absent in the profiles of the corresponding activated cells. In contrast, the predominant dihexose-terminated biantennary glycan at mass 2653 in activated CD8 and CD4 cells is a minor peak in the profiles from fresh cells. More detailed annotations of the MS spectra, using the cartoonist algorithm (35), are provided in Fig. S1 (supplemental data).

Further characterization of the major species by MS/MS analysis confirmed that the termini of the complex glycans were NeuGc-Hexose-HexNAc in the fresh cells and Hexose-Hexose-HexNAc in the activated cells (data not shown). Methylation analysis revealed that there was a significant change in the substitution of galactose at the 6 and 3 positions for N-glycans of fresh and activated (IL-2/IL-12) CD8 T cells (Table I). Although the methylation analysis comprises results from all glycans including high mannose glycans, galactose occurs only as a penultimate or terminal residue, so the substitution pattern of the galactose residues provides direct information about the terminal substitutions on complex N-linked glycans. Substitution of galactose at the 6 position went from 42 to 9% of the total galactose in fresh and activated cells, respectively, while substitution at the 3 position went from 11 to 53%. Taken together with the MALDI-TOF data showing loss of sialic acids and an increase in terminal he xoose, the data are consistent with the predominant terminal sequences on fresh and activated CD8 T cells as sequences previously reported on N-linked glycans of murine lymphocytes, NeuGcα2-6Galβ1-4GlcnAc (36, 37), and Gaα1-3Galβ1-4GlcnAc (38), respectively.

In marked contrast to the dramatic changes in the major complex type N-glycans of fresh and activated T cells, there was little difference in the major N-linked glycans of fresh and activated B cells. In both fresh and activated B cells, the predominant structures were biantennary capped with one or two NeuGc residues.

**Figure 2**. MALDI-MS profiles of N-glycans of fresh and activated T and B cells. Shown are comparisons of MALDI-MS profiles of N-linked glycans from fresh (A, C, and E) and activated (B, D, and F) T and B cells. CD8 T cells (A and B) and, CD4 T cells (C and D), were activated with anti-CD3 and IL-2/IL-12, B cells (E and F) were activated with anti-IgM and IL-4. Major peaks representing complex type N-glycans of either the fresh or activated cells are annotated with structures in symbol form. Dramatic changes are evident in the profiles of both the activated T cell populations, while little change is evident following activation of B cells. More detailed annotations are given in supplemental data (Fig. S1).

**Altered expression of terminal Galα1-3Gal and NeuGcα2-6Gal linkages in activated T cells**

To confirm that the Gaα1-3Gal sequence did indeed increase following activation of CD8 and CD4 T cells, FACS analysis was performed using the GS-I-B4 lectin from *G. simplicifolia*, which exhibits high specificity for this sequence. As shown in Fig. 3, there was a 10- to 30-fold increase in binding of the GS-I-B4 lectin in activated T cells relative to resting T cells. This observation is consistent with the dramatic increase in the Galα1-3Gal sequence detected in analysis of the N-linked glycans (Fig. 2 and Table I). Conversely, there was an activation-dependent decrease in the Siaα2-6Gal sequence in CD8 cells, as detected by the SNA lectin specific for this sequence. Although a significant change in SNA binding was not detected in CD4 cells, it is possible that the Siaα2-6Gal sequence is also present on glycoproteins with O-linked glycans, and/or glycolipids, which are turning over slower than glycoproteins that comprise the majority of N-linked glycans profiled in Fig. 2. No change in binding of either the GS-I-B4 or SNA lectins to activated B cells relative to resting B cells was observed. Taken together, these phenotypic results are consistent with the increase in the Galα1-3Gal linkage and a decrease in the NeuGcα2-6Gal sequences detected on activated T cells by direct analysis of N-linked glycans.

6 The online version of this article contains supplemental material.
IL-4 (39, 40). In particular, increased expression of these genes was not detected in CD4 cells cultured with IL-12, but consistent with a CD4 Th2 response. In contrast, expression of IL-13 genes was observed only in CD4 cells cultured with IL-4, consistent with further confirmation of the programmed activation resulting in vitro activation of CD4 cells with IL-12 or were consistent with the early (72 h) programmed changes in gene expression following in vitro activation of CD4 cells with IL-12 or IL-2/IL-12, or IL-2/IL-4) and B cells (anti-IgM with IL-2/IL-4) for a total of 30 separate RNA preparations. Labeled samples were prepared for each RNA and then hybridized to microarrays yielding three sets of data for fresh cells and each activation condition. Present and absent calls were assigned by the MAS 5.0 algorithm (Affymetrix), and a gene was deemed present if at least two of the three triplicate samples gave a present call (present call). Expression signals were generated using the RMA algorithm expressed in log 2 (30), and differences between expression in the fresh and activated cells were assessed for statistical significance and converted to “fold differences” for presentation.

The GLYCOv1 chip also contained cytokine genes which allowed further confirmation of the programmed activation resulting from cytokines present during activation. The changes observed were consistent with the early (72 h) programmed changes in gene expression following in vitro activation of CD4 cells with IL-2 or IL-4 (39, 40). In particular, increased expression of the IL-4 and IL-13 genes was observed only in CD4 cells cultured with IL-4, consistent with a CD4 Th2 response. In contrast, expression of these genes was not detected in CD4 cells cultured with IL-12, but expression of the IFN-γ gene is highest in these cells, consistent with the early stages of conversion to the Th1 subtype.

Samples were compared for expression of 122 glycosyltransferase and sulfotransferase genes by unsupervised hierarchical cluster analysis as shown in Fig. 4. Expressions of genes in fresh B and activated B cells and fresh CD4 and CD8 T cells are more closely related to each other than activated CD4 and CD8 T cells, which cluster separately. Of the 122 glycan transferase genes evaluated, 52 genes exhibited one or more statistically significant differences between activated cells and the corresponding fresh cells (see Materials and Methods for statistical analysis and Table S1 available as supplemental data for a complete listing of results for all genes). Of these, 23 showed differences of <1.5-fold, and 29 showed differences >1.5-fold. Of the 23 with differences <1.5-fold, 11 could be considered not meaningful (e.g., statistically significant differences between samples with all absent calls). However, for the 29 with differences >1.5-fold, only one would be considered not meaningful. Of these, only 13 of the genes code for enzymes that are potentially relevant to the biosynthesis of N-linked glycans, and the remainder are primarily relevant to the synthesis of other glycan classes including 5 for O-linked glycans, 4 for proteoglycans, 2 for glycolipids, 2 for glycophosphatidylinositol anchors, and 2 with undefined activity.

Of the two genes coding for enzymes involved in terminal glycosylation of O-linked glycans (Table II), one is ST3Gal I, which

Table I. Methylation analysis of N-linked glycans of fresh and activated (IL-2/IL-12) CD8 T cells

<table>
<thead>
<tr>
<th>Elution Time (Min)</th>
<th>Characteristic Fragment Ions</th>
<th>Assignment</th>
<th>Relative Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.73</td>
<td>102, 118, 129, 145, 161, 162, 205</td>
<td>Terminal galactose</td>
<td>0.09 0.12</td>
</tr>
<tr>
<td>19.92</td>
<td>101, 118, 129, 234</td>
<td>3-Galactose</td>
<td>0.02 0.17</td>
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<tr>
<td>20.43</td>
<td>118, 129, 145, 189, 233</td>
<td>6-Galactose</td>
<td>0.08 0.03</td>
</tr>
<tr>
<td>18.47</td>
<td>102, 118, 129, 145, 161, 162, 205</td>
<td>Terminal mannose</td>
<td>0.77 0.55</td>
</tr>
<tr>
<td>19.62</td>
<td>129, 130, 161, 190, 234</td>
<td>2-Mannose</td>
<td>1.62 0.42</td>
</tr>
<tr>
<td>20.02</td>
<td>118, 129, 145, 189, 233</td>
<td>6-Mannose</td>
<td>0.04 0.02</td>
</tr>
<tr>
<td>20.81</td>
<td>129, 130, 190, 233</td>
<td>2,4-Mannose</td>
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</tr>
<tr>
<td>21.19</td>
<td>129, 130, 189, 190</td>
<td>2,6-Mannose</td>
<td>0.01 0.04</td>
</tr>
<tr>
<td>21.36</td>
<td>118, 129, 189, 234</td>
<td>3,6-Mannose</td>
<td>0.27 0.42</td>
</tr>
<tr>
<td>16.95</td>
<td>102, 118, 131, 175</td>
<td>Terminal fucose</td>
<td>0.07 0.06</td>
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<tr>
<td>22.28</td>
<td>117, 143, 145, 159, 203, 205</td>
<td>Terminal GlcNAc</td>
<td>0.03 0.02</td>
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<tr>
<td>23.18</td>
<td>117, 159, 233</td>
<td>4-GlcNAc</td>
<td>1.00 1.00</td>
</tr>
<tr>
<td>24.02</td>
<td>117, 159, 346</td>
<td>3,4-GlcNAc</td>
<td>&lt;0.01 0.01</td>
</tr>
<tr>
<td>24.48</td>
<td>117, 159, 261</td>
<td>4,6-GlcNAc</td>
<td>0.05 0.09</td>
</tr>
</tbody>
</table>

Microarray analysis of the expression of cytokine and glycan transferase genes

For gene expression analysis, mRNAs were extracted from purified cell populations of fresh and activated lymphocytes and subjected to analysis on a custom Affymetrix-based DNA microarray containing murine and human glycosyltransferase, sulfotransferase, and cytokine genes, made available by the Consortium for Functional Glycomics. Three independent RNA preparations were made for each experimental condition comprising fresh and activated CD4 and CD8 T cells (anti-CD3 with IL-2, IL2/IL-12, or IL-2/IL-4) and B cells (anti-IgM with IL-2/IL-4) for a total of 30 separate RNA preparations. Labeled samples were prepared for each RNA and then hybridized to microarrays yielding three sets of data for fresh cells and each activation condition. Present and absent calls were assigned by the MAS 5.0 algorithm (Affymetrix), and a gene was deemed present if at least two of the three triplicate samples gave a present call (present call). Expression signals were generated using the RMA algorithm expressed in log 2 (30), and differences between expression in the fresh and activated cells were assessed for statistical significance and converted to “fold differences” for presentation.

FIGURE 3. FACS analysis of cell surface Galα1-3Gal (A) and Siaα2-6Gal (B) sequence in resting and activated lymphocytes. Splenocytes isolated from C57BL/6 mice were cultured with (thick line) or without (thin line) activator as described in Materials and Methods and the medium was supplemented with IL-2/IL-12 for the preparation of activated T cells. After 72 h in culture, resting or activated splenocytes were harvested and CD4, CD8 T cells, and B cells were purified as described in Materials and Methods. Purified lymphocyte cell populations were evaluated for expression of the terminal Galα1-3Gal and Siaα2-6Gal sequences by FACS analysis using GS-I-B 4 and SNA lectins, respectively, as described in Materials and Methods.
forms the O-linked glycan sequence NeuAc(Gc)α2-3Galβ1-3GalNAc and regulates the expression of PNA receptors in developing thymocytes (1, 2) and in resting and activated peripheral CD8 T cells (9) and CD4 T cells (10). Although there is only a decrease of 1.3- to 1.5-fold in the signal between fresh and activated T cells (and B cells), it is statistically significant, and appears to be sufficient to cause hyposialylation of O-linked glycan resulting in the PNA-high phenotype resulting from the increased expression of the Galβ1-3GalNAc ligand recognized by PNA (9, 10). The increase in the other enzyme, ST6GalNAc IV transferred, was previously noted by Kaufmann et al. (41) to be induced rapidly following activation of T cells and diminishing within 24 h.

**Glycan transferase changes relevant to N-linked glycans**

Of the results summarized in Table II, 13 genes relevant to the synthesis of N-linked glycans exhibited significant changes in gene expression. Seven of these are involved in the synthesis of elements of N-linked glycan structure common to all cells because they either 1) participate in the synthesis of the high mannose lipid-linked intermediate (mannosyltransferases), 2) are subunits of oligosaccharyltransferase that transfers the glycan to asparagine (DAD1, ribophorin I and II), or 3) form the ubiquitous Galβ1-4GlcNac sequence that extends the branches of most complex type N-linked glycans (b4GalT1). Consistent with the constitutive expression of N-linked glycans in all cells, these genes are expressed in all resting and activated cell populations. The six genes involved in the synthesis and transfer of the lipid-linked intermediate in general showed increased expression in activated T cells, with 25 of 42 comparisons showing significant 1.5- to 2.9-fold increases in signal, consistent with the increased need for N-linked glycans in proliferating cells.

The remaining six genes are involved in the synthesis of terminal sequences in N-linked (and O-linked) glycans. In contrast to the genes involved in the synthesis of the core N-linked glycans, these genes were differentially regulated, showing both positive and negative changes in expression (Table II and Fig. 5). Of these, the β-galactoside α2-6 sialyltransferase (ST6Gal I) and β-galactoside α1-3 galactosyltransferase (α1-3GalT) are responsible for the synthesis of the terminal sequences NeuGcα2-6Galβ1-4GlcNAc (42) and Galα1-3Galβ1-4GlcNAc (43), respectively, that account for the major changes in terminal glycosylation of N-linked glycans.

**Table II. Expression fold changes in glycosyltransferase expression in resting and activated lymphocytes**

<table>
<thead>
<tr>
<th>Subcategory</th>
<th>CD4 Activation Condition</th>
<th>CD8 Activation Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subcategory</td>
<td>CD4 Present Cells (Fresh: Activated)</td>
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<td></td>
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<td>Sialyltransferase</td>
<td>ST6Gal IV</td>
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<td>Sulfotransferase</td>
<td>GlcNAcST-1</td>
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Changes in the expression levels in fresh and activated CD8 T cells with IL-2/IL-12. Each enzyme is identified by its common name and the symbol linked (or O-linked) glycans, with the sugar transferred by the enzyme identified by an asterisk (*). Each enzyme exhibits a unique pattern of expression relative to the others with respect to positive or negative regulation upon activation, and differential expression in T and B cells. Comparisons with other enzymes and activation conditions are summarized in Table II.

The changes in expression of the other four genes do not appear relevant to the major changes seen in the N-glycan MALDI-TOF MS profiles documented in Fig. 2. The ST8Sia IV enzyme exhibits a dramatic decrease following activation of all three lymphocyte types. ST8Sia IV is a polysialyltransferase that transfers one or more sialic acid residues in the [NeuAc(Gc)a2-8]nNeuAc(Gc)a2-3Gal sequence on N-linked glycans of a limited number of glycoproteins including neural cell adhesion molecule (45). To date, such sialylated structures have not been reported on lymphocytes, and while they may be present in small amounts, they do not comprise an observable component of the N-glycans analyzed in this report. Two enzymes that synthesize the sequence NeuAc(Gc)a2-3Galβ1-4GlcNAc on N-linked and O-linked glycans, ST3Gal IV and ST3Gal VI (46–48), were differentially expressed in T cells. ST3Gal IV went from absent to present, and ST3Gal VI went from present to absent (Table II). The increase in expression of ST3Gal IV has been previously reported in activated CD4 cells following 6–10 days of activation (11) and is believed to play a critical role in the formation of the sialyl-Lewis X linkage on O-linked glycans that serve as ligands for selectins (11, 49). However, the dramatic reduction in sialylated N-glycans in activated CD4 and CD8 T cells (Fig. 2) indicates that ST3Gal IV is not a major contributor to terminal glycosylation of N-glycans. Although a decrease in the expression of the ST3Gal VI enzyme is consistent with loss of sialylated glycans (Fig. 2), it does not account for the net loss in 6-substituted galactose and increase in 3-substituted galactose. Finally, the GlcNAc6ST-1 is a sulfotransferase that forms the Galβ1-4(6S)GlcNAc sequence. Although sulfated structures are not captured in the MALDI-TOF profiling protocols used for analysis of N-glycans in this report and could have been missed, the product of this enzyme has been shown to be expressed primarily on high endothelial venules of lymph nodes and not on lymphocytes (50, 51).

Discussion

Comparison of the N-glycan structures following activation of T and B lymphocytes has revealed a heretofore unrecognized change in terminal glycosylation of N-linked glycans of activated murine T lymphocytes involving the loss of charged sialylated glycans terminated with the NeuGcα2-6Gal sequence, and an increase in uncharged glycans terminated with the Galα1-3Gal sequence. This decrease in RMA signal, the opposite from T cells. These results are consistent with the observed retention of N-linked glycans with the terminal NeuGcα2-6Gal sequence in both resting and activated B cells (Fig. 2).

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change corresponds to changes in expression of the two glycosyltransferases responsible for their synthesis, a decreased expression of the sialyltransferase ST6Gal I, and increased expression of the galactosyltransferase α1-3GalT, respectively. In contrast to the change observed in T cells, B cells maintain their sialylated N-linked glycans following activation, consistent with increased expression of ST6Gal I and decreased expression of α1-3GalT.

Reports from other laboratories corroborate aspects of these findings for regulation of the ST6Gal I gene in activated lymphocytes in vivo. In particular, using an in vivo transgenic lymphocytic choriomeningitis virus model for Ag activation of CD8 cells, Kaech et al. (26) found ST6Gal I to be one of the genes that was down-regulated in CD8 effector cells 8 days after transfection. Moreover, effector cells exhibited reduced binding of the SNA that recognizes the Siaα2-6Gal sequence (Ref. 52, and L. Baum, T. Onami, and R. Ahmed, unpublished observations). This study recapitulates in vivo the results obtained with CD8 cells in this report. Although only modest reduction of SNA binding was observed comparing native and activated CD4 cells in this study, this may be due to the fact that the residual sialylated glycans at the end of 72 h are sufficient to support significant lectin binding to other glycan classes (e.g., glycolipids). In murine B cells, increased expression of the ST6Gal I gene has been observed, mediated by a B cell-specific promoter (53). Although peak expression occurs 10 days after activation, significant increases in expression were observed within 72 h as observed in this report. (Probe sets for one of two alternatively spliced forms of ST6Gal I are shown in Table II. The probe set corresponding to the “long form” is derived from the 3′ end of a 4.3-kb mRNA, while the probe set for the “short form” is at the 3′ end of a ~2-kb mRNA resulting from an alternative polyA addition site 2 kb from the 3′ end of the 4.3-kb transcript. Although 4.3-kb message is the predominant mRNA for this gene in most human tissues (48), significant up-regulation of the “short form” was seen in B cells (see supplementary material)).

The loss of the NeuGcα2-6Gal sequence has functional implications relevant to the interaction of T cells and B cells with glycan-binding proteins. The B cell membrane marker CD22 is well-known as a regulator of BCR signaling, and is a member of the Siglec family (Siglec-2) with an extracellular N-terminal Ig domain that recognizes the NeuGcα2-6Gal sequence as a preferred ligand on murine cells (54, 55). CD22 interacts with glycoprotein ligands on the same cell, in cis, and on adjacent cells, in trans. Interaction of CD22 with B cell ligands in cis has been suggested to modulate the activity of CD22 as a negative regulator of B cell signaling (23, 25, 56, 57). However, CD22 was initially described as a T cell adhesion protein and has been demonstrated to interact with trans ligands on adjacent cells (23, 58, 59). Thus, differential regulation of the NeuGcα2-6Gal sequence may modulate CD22 function through its complex interaction with cis and trans ligands, particularly in B-T cell interactions (e.g., B cell Ag presentation to T cells). The ST6Gal I transferase expression has also been proposed to modulate binding of galectin-1 to CD45 on T cells leading to cell death (19). In this case, sialylation of CD45 glycans blocks the terminal Galβ1–4GlcNAc-binding sites used as a ligand by galectin-1. Thus, reduced ST6Gal I expression in activated T cells could lead to increased binding of galectin-1. In this regard, following activation of CD4 and CD8 T cells, we observed significant enhancement of the binding of Erythrina cristagalli agglutinin, which binds the Galβ1–4GlcNAc sequence (data not shown).

Concomitant with a loss of the NeuGcα2-6Gal sequence, N-linked glycans of activated T cells acquire the Galα1-3Gal sequence, the product of the galactosyltransferase, α1-3GalT. In humans, this enzyme is catalytically inactive, and consequently the Galα1-3Gal sequence is not expressed. It will be of interest to assess the glycosylation changes in human lymphocytes to see whether the same loss of sialylated glycans occurs, and if so, what if any sequence replaces the Galα1-3Gal sequence in murine cells.

With the increasing attention of the roles of glycan-binding proteins in immune function, the paucity of information on glycan structures on leukocytes has become increasingly evident, and is a limiting factor in elucidating the biology they mediate (60–64). The approach used in this report has general use for detecting changes in glycosylation of closely related leukocyte cell populations (e.g., pre-/postdifferentiation or activation, immature/mature, etc.) and establishing the biosynthetic basis for those changes. With present technology, the MALDI-TOF MS and supporting MS/MS and methylation analysis experiments provide meaningful data on the most abundant glycans. However, some subsets of glycans, for example sulfated structures, would be missed using protocols described in this report. Perhaps even more important, literally hundreds of minor glycans can be detected by MS analysis, but the subsequent annotation and verification of structure is prohibitive. However, progress for developing automatic methods of annotation with sophisticated algorithms is being made (65–67), and technology advances in sample preparation and analysis will rapidly evolve.

Combining glycan structure analysis and global expression analysis using oligonucleotide microarrays may provide increasingly rich information about the regulation of glycosylation of pure cell populations responding to environmental signals such as those studied here. It is estimated that there may be a total of ~250 glycan transferase genes in the murine (and human) genomes. Although the microarray used for this study (GLYCoV1 chip) contained 122 unique murine glycosyltransferase genes, an updated version, GLYCoV3, contains ~200 murine glycan transferase genes, providing more complete coverage of the biosynthetic pathways. An inherent problem with microarray data is that some probe sets “do not work”. Two genes in particular, FucT-VII and core 2 GlcNAc transferase, known to be involved in Sialyl-Lewis X selectin ligand formation on O-linked glycans of CD4 T cells (11–16, 68), were not detected (see supplemental data). However, our recent experience is that redesign of the probe sets can largely remedy this problem and should lead to increasingly powerful data sets. Even with a complete set of genes, changes detected in gene expression may not be reflected in major changes in glycan structure (e.g., changes in ST3Gal VI and ST8Sia IV in this report), because not all enzymes will be rate limiting, and some may be expressed at levels below the threshold needed to compete with other enzymes in the pathway. However, recent efforts to develop algorithms that take global microarray data to predict glycan structure show promise (69), and could evolve, through information gleaned from data sets of expression data from cells with known glycan profiles, to recognize significant from insignificant changes in gene expression. Current efforts to combined advances in glycan structure analysis and technologies for functional gene expression analysis are likely to converge to provide a systems biology approach to analysis of glycan structure and function that will facilitate future investigations into roles of glycan-binding proteins in immune cell function (70).

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Disclosures
The authors have no financial conflict of interest.


