Background: The functional relevance of ST6Gal-I down-regulation during monocyte activation/macrophage differentiation is not well understood.

Results: Cell and transgenic mouse models suggest that ST6Gal-I-mediated sialylation of TNFR1 blocks TNFα-induced apoptosis.

Conclusion: ST6Gal-I down-regulation may limit monocyte/macrophage lifespan by sensitizing cells to apoptosis via TNFR1 hyposialylation.

Significance: This is the first determination that TNFR1 function is regulated by its glycan structure.

Macrophages play a central role in innate immunity, however mechanisms regulating macrophage survival are not fully understood. Herein we describe a novel apoptotic pathway involving α-2-6 sialylation of the TNFRI death receptor by the ST6Gal-I sialyltransferase. Variant glycosylation of TNFRI has not previously been implicated in TNFRI function, and little is known regarding the TNFRI glycan composition. To study sialylation in macrophages, we treated U937 monocytic cells with PMA, which stimulates both macrophage differentiation and apoptosis. Interestingly, macrophage differentiation induces ST6Gal-I down-regulation, leading to reduced α-2-6 sialylation of selected receptors. To prevent loss of α-2-6 sialylation, we forced constitutive expression of ST6Gal-I, and found that this strongly inhibited PMA-induced apoptosis. Given that PMA-mediated apoptosis is thought to result from up-regulation of TNFα, which then activates TNFRI, we next evaluated the α-2-6 sialylation of TNFRI. U937 cells with forced ST6Gal-I displayed TNFRI with elevated α-2-6 sialylation, and this was associated with diminished TNFα-stimulated apoptosis. Correspondingly, removal of α-2-6 sialylation from TNFRI through either neuraminidase treatment or expression of ST6Gal-I shRNA markedly enhanced TNFα-mediated apoptosis. To confirm the physiologic importance of TNFRI sialylation, we generated over-expressing ST6Gal-I transgenic mice. Peritoneal macrophages from transgenic lines displayed TNFRI with elevated α-2-6 sialylation, and these cells were significantly protected against TNFα-stimulated apoptosis. Moreover, greater numbers of thioglycolate-induced peritoneal cells were observed in transgenic mice. These collective results highlight a new mechanism of TNFRI regulation, and further intimate that loss of α-2-6 sialylation during macrophage differentiation may limit macrophage lifespan by sensitizing cells to TNFα-stimulated apoptosis.

The sialyltransferase family of glycosyltransferases catalyzes the addition of the negatively charged sugar, sialic acid, to various glycoconjugates on glycoproteins and glycolipids (1, 2). The trans-Golgi ST6Gal-I2 sialyltransferase (β-galactoside α-2-6-sialyltransferase-1) adds an α-2-6-linked sialic acid to the termini of N-linked glycans on glycoproteins bound for the plasma membrane or secretion (3). The importance of ST6Gal-I in the immune system was established by the finding that ST6Gal-I-deficient mice have impaired thymopoiesis and granulopoiesis (4, 5), as well as defective B cell maturation and antibody production (6). Intriguingly, ST6Gal-I expression is dynamically regulated in some immune cell populations. For example, our group has reported that ST6Gal-I is down-regulated during the differentiation of monocytes along the macrophage lineage, which in turn leads to the loss of α-2-6 sialylation from selected cell surface receptors including the β1 integrin (7–9).

To study the role of ST6Gal-I in monocyte behavior we previously employed a standard in vitro model system. Specifically, U937 promonocytic cell lines were treated with the protein kinase C (PKC) activator, phorbol-12-myristate-13-acetate (PMA), which is known to stimulate monocyte activation as well as macrophage differentiation. These experiments revealed that PMA, acting through a PKC/ras/ERK signaling pathway, induced up-regulation of the BACE1 β-secretase, which then directed cleavage and shedding of ST6Gal-I from cells (9). ST6Gal-I was also reported by Taniguchi et al. (10) to be down-regulated through transcriptional mechanisms in PMA-treated HL60 myeloid cells. We hypothesize that shedding and transcriptional repression cooperate to maintain low levels of ST6Gal-I (and consequently α-2-6 sialylation) in differentiated cells. Importantly, primary human CD14+ monocytes differentiated to macrophages using human serum rather than...
PMA also exhibited ST6Gal-I down-regulation (9), indicating that loss of ST6Gal-I-dependent α2-6 sialylation is associated with cell differentiation, and not an isolated effect of PMA. Taken together these results suggest that ST6Gal-I down-regulation may be critical for some aspect of the monocyte/macrophage phenotype.

In addition to regulating cell differentiation, PMA treatment of monocytes induces apoptosis (11–14), therefore in the current study we evaluated whether ST6Gal-I down-regulation had an effect on death receptor signaling. It has been shown that PMA-directed apoptosis occurs as a consequence of increased PKC-dependent expression and secretion of tumor necrosis factor α (TNFα), which then binds TNF receptors (TNFR) to induce cell death (12–16). There are two principal receptors that bind TNFα, TNFR1, and TNFR2. These receptors are part of a larger TNFR superfamily that also includes Fas (CD95) and TRAIL-R (DR4 and DR5). Only TNFR1 is considered a canonical death receptor because TNFR2 does not contain the death domain (17, 18). Engagement of TNFR1 with TNFα can give rise to two opposing biological outcomes, survival and apoptosis, depending upon the formation of distinct intracellular signaling complexes on the TNFR1 cytosolic domain.

Monocyte/macrophage apoptosis plays a key role in many physiologic and pathophysiologic processes including atherosclerosis and immune disorders. Signaling through TNFR1 represents a predominant apoptotic pathway in macrophages, however the underlying mechanisms that regulate the balance between macrophage survival and apoptosis remain ill-defined. Although intensive research has been directed at understanding aberrant TNFR1 signaling, the contribution of variant glycosylation to TNFR1 function has not previously been addressed. In the current study we report that TNFR1 is a substrate for ST6Gal-I in both human monocytes and primary macrophages harvested from overexpressing ST6Gal-I transgenic mice. More importantly, our results show that ST6Gal-I-mediated α2-6 sialylation of TNFR1 inhibits apoptosis, thus suggesting a new paradigm in death receptor signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Lines**—Human monocytic U937 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 1% amphotericin B, and 1% penicillin/streptomycin. Cells constitutively expressing either ST6Gal-I (ST6) or empty vector (EV) were established using a lentiviral vector, and stably transduced cells were obtained and characterized as previously reported (9). Endogenous ST6Gal-I in the parental U937 cells (Par) was knocked down by introducing two different shRNA sequences using lentiviral vectors from Sigma-Aldrich. The cells were selected by addition of 10 μg/ml of puromycin (Sigma-Aldrich), and then maintained in 0.5 μg/ml puromycin. The puromycin was removed from the medium at least 2 days in advance of all experiments. Each of the established cell lines used for these studies represents a pooled population of stably transduced clones.

**PMA and TNFα Induced Apoptosis**—U937 cells were seeded in 6-well plates (ultra low attachment, Corning Inc.) in complete DMEM medium overnight. Following this incubation, cells were treated for 24 h with either 100 ng/ml of PMA (Sigma-Aldrich) or a combination of 10 ng/ml TNFα (R&D Systems) plus 5 μM cycloheximide (CHX, from Sigma-Aldrich). Cells were then analyzed for apoptosis by using flow cytometry or FLICA staining as described below.

**Neuraminidase Treatment**—Cells were incubated with 100 units of Clostridium perfringens neuraminidase (New England Biolabs) in 50 mM sodium citrate buffer (pH 6.0) for 1 h at 37 °C. The treated lysates were then immunoblotted for TNFα. To test whether sialylation status affects cell apoptosis, 5 × 10^5 cells were treated with or without 100 units of neuraminidase in PBS (pH 7.4) for 1 h at 37 °C. After washing three times with PBS, cells were treated with or without 10 ng/ml TNFα plus 5 μM cycloheximide for 24 h, and then evaluated for apoptosis.

**Flow Cytometric Analysis for Apoptosis**—Cells were stained with FITC annexin v and PI using the Vybrant Apoptosis Assay kit (Molecular Probes) for 15 min. Stained cells were analyzed with FACSCalibur (Becton Dickinson).

**Immunofluorescent Staining for Activated Caspases**—Cells were stained for activated caspases 3 and 7 following the protocols of the sulforhodamine FLICA apoptosis detection kit (Immunochemistry Technologies). Nuclei were labeled by Hoechst stain for 5 min. The cells were then seeded onto slides and viewed under a fluorescent microscope (Nikon). Images were taken by a Nikon CoolSNAP camera. The number of FLICA-positive cells was quantified by counting cells from multiple fields.

**ELISA Assay for TNFα**—Cells treated with or without PMA were cultured for 24 h, and the culture supernatants were subsequently collected. The amount of TNFα in the media was measured using the human TNF ELISA kit II from BD Biosciences.

**Western Blotting**—Cells were harvested and lysed in 50 mM Tris buffer (pH 7.4) containing 1% Triton X-100 and a protease inhibitor mixture (Roche Applied Science). Protein concentrations were quantified by Bio-Rad assay. Lysates were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk and then incubated with a primary antibody, followed by an HRP-conjugated secondary antibody, and detection was accomplished using the Immobilon Chemiluminescent HRP substrate (Millipore). Human and mouse TNFR1 were detected using a rabbit monoclonal antibody from Cell Signaling, and a polyclonal antibody from Abcam, respectively. V5 was immunoblotted with a monoclonal antibody from Sigma-Aldrich. ST6Gal-I expression in mouse primary macrophages was detected with a rabbit polyclonal antibody from Santa Cruz Biotechnology, whereas ST6Gal-I expression in U937 cells was detected using a mouse monoclonal antibody from Sigma-Aldrich or a goat polyclonal antibody from R&D Systems.

**SNA and MAA Precipitation**—500 μg of cell lysates were incubated overnight at 4 °C with either 60 μl of SNA-conjugated agarose beads (Vector Laboratories) or MAA-conjugated agarose beads (EY Laboratories). α2-6 or α2-3-sialylated proteins bound to the beads were precipitated by centrifugation and washed extensively with lysis buffer. The sialoprotein/lec-
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tin conjugates were then resolved by SDS-PAGE and immunoblotted for TNFR1 as described previously.

PNGaseF Treatment—30 μg of cell lysates were incubated with 2 μl of Flavobacterium meningosepticum PNGaseF (New England Biolabs) at 37 °C for 1 h according to manufacturer’s instructions. The treated lysates were then immunoblotted for TNFR1. To confirm whether TNFR1 sialylation is through α2-6-linked N-glycans, 250 μg of cell lysates were digested with 10 μl of PNGaseF at 37 °C for 4 h. The lysates were then precipitated by SNA lectin, and precipitates were immunoblotted for TNFR1.

ST6Gal-I Transgenic Mice and Genotyping—A rat ST6Gal-I gene engineered into a pcDNA 3.1/V5-His vector, driven by a CMV promoter, was initially obtained from Dr. Karen Colley (University of Illinois, Chicago). The CMV promoter/V5-epitope-tagged ST6Gal-I transgenic construct with BGH polyadenylation signal was excised from the plasmid backbone and injected into C57BL/6 embryos. Two strains (Line 1 and Line 2) from two independent founder mice were maintained for the studies. Genotyping was used to select the mice carrying the rat ST6Gal-I gene. The sequences of the primers for the genotyping are as follows: T7 forward-CTATAGGGAGACCAAGCTG and ST6 reverse-TCAGAGGCTAGGTAAAC-GAC. The product of the PCR reaction was excised from gels and sequenced to confirm integration of the ST6Gal-I transgene. Age- and gender-matched C57BL/6 wild-type mice were used as controls for experiments in this study. All experiments involving wild-type and transgenic mice were performed with prior approval from the UAB Institutional Animal Care and Use Committee (IACUC).

Isolation and Apoptosis Analysis of Peritoneal Macrophages from Mice—A 4% thioglycollate solution in distilled water (Sigma-Aldrich) was autoclaved for 15 min. To elicit an inflammatory response and macrophage emigration, 1 ml of the thioglycollate solution was injected intraperitoneally into 8–10-week-old mice. Four days later, the mice were sacrificed and peritoneal macrophages were collected by flushing the peritoneal cavity with ice-cold and serum-free DMEM. After washing with DMEM, the cells were re-suspended in 1 ml of medium. 10 μl of cells were placed into 990 μl of 2% acetic acid, and then 10 μl of cells in the acetic acid solution were placed on a hemacytometer, and cell number was quantified. The acetic acid lyses the cell membrane while keeping the nuclear membrane intact so that the cell counts represent nucleated cell numbers.

2 × 10^5 freshly-harvested peritoneal cells were stained with FITC annexin V and PI using the Vybrant Apoptosis Assay kit, and then evaluated for apoptosis by flow cytometry. The remaining cells were then resuspended in DMEM supplemented with 10% FBS, and seeded at a density of 5 × 10^5 cells/cm^2. After incubating for 3 h to allow attachment, the media was replaced with fresh medium to remove non-adherent cells and attached macrophages were cultured overnight. The cells were then treated with 10 ng/ml TNFα plus 5 μM CHX for 24 h and stained for apoptosis using Vybrant Apoptosis Assay kit.

RESULTS

ST6Gal-I Prevents PMA-induced Apoptosis in U937 Cells—To better understand the role of ST6Gal-I in monocyte/macrophage behavior, we previously generated a stable U937 cell line that constitutively expresses ST6Gal-I (9). As shown in Fig. 1A, treatment of Par or EV U937 cells with PMA causes down-regulation of the endogenous ST6Gal-I enzyme. However, cells with forced expression of ST6 have increased basal levels of enzyme (representing the combined endogenous and exogenous ST6Gal-I), and high levels of ST6Gal-I are maintained following PMA treatment.

PMA causes monocytic cells, including U937 cells, to exit the cell cycle, begin differentiating along the macrophage lineage, and ultimately undergo apoptosis. Accordingly, we evaluated the effects of ST6Gal-I activity on cell proliferation and survival. Cells were suspended in media with or without PMA, and then seeded into tissue culture dishes at a density of 2 × 10^5. Twenty-four hours later, there was a 2-fold increase in cell number for Par and EV U937 cells in control media, which is consistent with the known rapid doubling time of U937 cells (Fig. 1B). Treatment of Par and EV cells with PMA completely prevented this cell doubling, suggesting exit from the cell cycle and/or decreased cell survival. Remarkably, the ST6 cell population continued to expand in the presence of PMA, suggesting that ST6Gal-I-mediated sialylation may play some role in the shift between a proliferative versus differentiated phenotype.

Although the results in Fig. 1 suggested that ST6Gal-I likely affects both proliferation and cell survival, we focused on apoptosis in the current study because many of the molecular events in PMA-mediated apoptosis are known. To evaluate whether ST6Gal-I activity influences cell death, we performed immunostaining to detect activated caspases 3 and 7 (Fig. 2, A and B). As compared with untreated cells, a substantially greater number of apoptotic cells was apparent in Par and EV cells treated with PMA. In contrast, no significant difference in
the number of apoptotic cells was observed for untreated and PMA-treated ST6 cells.

Inhibition of PMA-induced apoptosis by ST6Gal-I was further confirmed by monitoring reactivity of the cells to annexin V and propidium iodide (PI). As shown in Fig. 2, C and D, PMA treatment of Par and EV cells caused the apoptotic rates to reach ~20–25%, whereas PMA-induced apoptosis was markedly attenuated in ST6 cells.

**Forced Expression of ST6Gal-I Blocks PMA-induced Loss of Sialylation from TNFR1**—PMA-mediated apoptosis of U937 cells is thought to be due to PMA-induced synthesis and secretion of TNFα, which then directs cell death by signaling through the TNFR1 receptor (12–15). To test whether ST6Gal-I activity alters TNFα expression, cells were treated with or without PMA for 24 h, and then culture supernatants were collected and TNFα production was monitored by ELISA. As shown in Fig. 3A, TNFα production was increased by about 4.5-fold following PMA treatment in all three cell lines, with no significant difference in levels observed. These results indicate that ST6Gal-I-mediated inhibition of apoptosis is not due to changes in TNFα synthesis or release.

We next tested whether ST6Gal-I activity caused greater α2-6 sialylation of TNFR1, leading to altered receptor signaling. To measure TNFR1 sialylation levels, cell lysates were incubated with agarose-conjugated SNA lectin, which binds specifically to α2-6 sialylated proteins. Sialylated proteins were then precipitated and immunoblotted for TNFR1. As shown in Fig. 3B, the highest expression level of α2-6 sialylated TNFR1 was observed in the untreated ST6 cells, although a considerable amount of α2-6 sialylated TNFR1 was observed in untreated Par and EV cells as well. Upon PMA treatment, levels of sialylated TNFR1 were dramatically reduced in Par and EV cells,
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FIGURE 3. PMA-induced down-regulation of ST6Gal-I is associated with loss of α2-6 sialylation from TNFR1. A, cells were treated with or without PMA for 24 h, and then the amount of TNFα secreted into culture media was measured by ELISA. Data are plotted as fold TNFα induced by PMA as compared with control samples. Values represent means and S.E. from three independent experiments. B, lysates from cells treated with or without PMA were incubated with agarocone-conjugated SNA lectin. α2-6 sialylated proteins were precipitated and blotted for TNFR1. C, total TNFR1 levels were detected by blots of whole cell lysates. D, cell lysates were treated with neuraminidase at 37 °C for 1 h, and then Western blot was performed with anti-TNFR1 antibody.

Consistent with PMA-induced down-regulation of endogenous ST6Gal-I, in contrast, TNFR1 from PMA-treated ST6 cells still retained substantial reactivity toward SNA, reflecting high α2-6 sialylation. These results suggest that TNFR1 is modified by ST6Gal-I, and that PMA induces expression of a hyposialylated TNFR1 isoform, Par, EV, and ST6 cell lysates were treated with neuraminidase, and then lysates were incubated with agarose-conjugated SNA lectin. α2-6 sialylated proteins were precipitated and blotted for TNFR1.

Total TNFR1 levels (sialylated + unsialylated) were also monitored by Western blots of whole cell lysates (Fig. 3C). Interestingly, two bands were observed for TNFR1 in untreated Par and EV cells, and the upper band was lost in these cells following PMA treatment. These data suggested that the upper band represents the α2-6 sialylated form of TNFR1. In contrast to Par and EV cells, the upper band was retained in PMA-treated ST6 cells, consistent with the expression of α2-6 sialylated TNFR1.

To further confirm that the upper band represents an α2-6 sialylated TNFR1 isofrom, Par, EV, and ST6 cell lysates were treated with neuraminidase and then Western blotted for TNFR1 (Fig. 3D). The upper band was eliminated by neuraminidase treatment in all three cell lines, confirming the identity of this band as a sialylated TNFR1 variant. These collective results are the first to identify TNFR1 as an ST6Gal-I substrate, and to show that α2-6 sialylation of TNFR1 is lost upon differentiation of monocytic cells along the macrophage lineage.

FIGURE 4. N-glycans are the major source of α2-6 sialic acids on TNFR1. A, cell lysates were treated with or without PNGase F, and then TNFR1 immunoblots were performed. B, cell lysates were treated with or without PNGase F, and then lysates were incubated with agarose-conjugated SNA lectin. α2-6 sialylated proteins were precipitated and blotted for TNFR1.

To determine whether α2-6 sialic acids were present on N-glycans, cell lysates were pretreated with PNGase F enzyme to remove N-linked glycosylation, and then TNFR1 was characterized. Immunoblots of TNFR1 in the PNGase F-treated lysates revealed a single band at ~45kD, indicating effective cleavage by PNGase F (Fig. 4A). The PNGase F-treated and control cell lysates were then incubated with SNA-agarose to precipitate α2-6 sialylated TNFR1 (Fig. 4B). TNFR1 was precipitated by SNA from control lysates, however in contrast, no detectable TNFR1 was observed in precipitates from PNGase F-treated lysates. These results suggest that the major source of α2-6 sialic acids on TNFR1 is N-linked, rather than O-linked, glycans.

α2-6 Sialylation of TNFR1 Controls TNFα-directed Apoptosis—To evaluate whether altered α2-6 sialylation of TNFR1 regulates apoptotic signaling, Par, EV, and ST6 cells were treated with TNFα and then monitored for annexin V reactivity. As shown in Fig. 5A, ST6 cells exhibited significantly less TNFα-mediated apoptosis than Par or EV cells. Notably, α2-6 sialylation is present on TNFR1 expressed by Par and EV (in the absence of PMA treatment), however the level of TNFR1 α2-6 sialylation in ST6 cells is clearly higher (see Fig. 3B). These results suggest that α2-6 sialylation of TNFR1 has a protective effect on cell death.

We next examined whether the forced loss of α2-6 sialylation from TNFR1 would sensitize cells to TNFα-mediated apoptosis. To this end, Par cells were treated with neuraminidase to remove sialic acids, and apoptotic rates were analyzed by annexin-V reactivity. TNFα-induced apoptosis was substantially increased in the cells treated with neuraminidase, indicating that reduced α2-6 sialylation of TNFR1 makes cells more susceptible to apoptosis (Fig. 5B).

To more directly investigate whether loss of α2-6 sialylation from TNFR1 promotes cell apoptosis, endogenous ST6Gal-I was knocked down in parental cells using shRNA. Two different shRNA sequences were tested, and for each a pooled population of stable clones was generated. As shown in Fig. 5C, ST6Gal-I levels were greatly reduced in both of the shRNA-
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FIGURE 5. Decreased α2-6 sialylation of TNFR1 promotes U937 cell apoptosis. A, cells were treated with or without TNFα plus CHX for 24 h (C, control). Cells were then analyzed by flow cytometry for annexin V and PI. Values represent means and S.E. for three independent experiments. B, annexin V and PI reactivity were analyzed in Par cells treated with neuraminidase at 37 °C for 1 h, followed by treatment with TNFα plus CHX for 24 h. C, Par cells were transduced with two different shRNA sequences (#1 and #2) against ST6Gal-I. Western blotting was performed to confirm knockdown. D, Par, EV, and shRNA-transduced cells were treated with TNFα plus CHX for 24 h, and then evaluated for annexin V and PI reactivity. E, lysates from Par, EV, ST6, or shRNA (sequence #2) cells were incubated with MAA-agarose and α2-3 sialylated proteins were precipitated by centrifugation. Precipitates were immunoblotted for TNFR1.

TNFRI Has Increased α2-6 Sialylation in Cells from Overexpressing ST6Gal-I Transgenic Mice—To elucidate the physiologic importance of ST6Gal-I in regulating TNFR1-mediated apoptosis, we generated transgenic mice that constitutively express ST6Gal-I. Briefly, a V5-tagged rat ST6Gal-I cDNA, cloned into the pcDNA 3.1/V5 vector that contains the CMV promoter (Fig. 6A), was injected into fertilized C57BL/6 eggs. Two independent transgenic lines were identified and studied (Line 1 and Line 2). Expression of the transgene was evaluated by immunoblotting. As shown in Fig. 6B, splenic homogenates from transgenic mice were positive for both V5 and ST6Gal-I, verifying protein expression of the transgene. The transgenic mice were fertile and did not present with any gross phenotypic abnormalities. Also, histologic analyses of multiple organs from older (>9 months) transgenic animals did not reveal any obvious differences from non-transgenic littermate controls.

To assess expression of ST6Gal-I in immune cells of transgenic mice, animals were injected intraperitoneally with 4% thioglycollate, and infiltrating cells were collected by lavage 96 h following injection. At 96 h, cells of the monocyte/macrophage lineage represent the predominate cell type within peritoneal infiltrates (19, 20). Peritoneal cells collected from 4 different transgenic mice exhibited elevated ST6Gal-I relative to wild-type mice (Fig. 6C), although the level of ST6Gal-I up-reg-
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ulation was relatively modest in this cell type. It is possible that this may reflect cell-type specific differences in the efficacy of the CMV promoter, and it should also be noted that transgene expression is hemiallelic. Nonetheless, SNA analyses (Fig. 6D) revealed that the TNFR1 expressed by transgenic cells had increased levels of α2-6 sialylation. Interestingly, immunoblots of TNFR1 expressed by mouse macrophages revealed only one band for the receptor (Fig. 6E), whereas two bands were observed in lysates from human U937 cells, with the upper band representing the sialylated form. We hypothesize that the size of variantly glycosylated TNFR1 isoforms in transgenic mouse cells may be too small to resolve by SDS-PAGE, because of more modest changes in the degree of sialylation.

Macrophages from Transgenic ST6Gal-I Mice Are Resistant to TNFα-induced Apoptosis—To examine whether forced ST6Gal-I-mediated sialylation of TNFR1 prevents monocyte/macrophage apoptosis in vivo, peritoneal cells were collected at 96 h following thioglycollate injection and evaluated for annexin reactivity. Intriguingly, an increased number of peritoneal cells was observed for each of the two independent transgenic mouse lines (Fig. 7A), potentially reflecting greater survival of these cells (although other mechanisms are also possible). Furthermore, cells from the transgenic animals displayed decreased annexin V reactivity (Fig. 7B), which may be due to reduced apoptosis induced by endogenous TNFα or other factors within the inflammatory milieu (e.g. galectins). To more directly assess TNFR1-mediated apoptosis in macrophages, peritoneal cells were cultured overnight, and then cells were treated with TNFα (Fig. 7, C and D). These experiments revealed that TNFα-induced apoptosis was also significantly attenuated in macrophages harvested from transgenic mice.

These results provide strong evidence that ST6Gal-I mediated α2-6 sialylation of TNFR1 plays a critical role in regulating apoptotic signaling from this receptor.

DISCUSSION

The TNFα/TNFR signaling axis plays a critical, but complex, role in the pathogenesis of chronic inflammatory and autoimmune disorders. The TNFR1 receptor can initiate both proliferative and apoptotic signaling cascades, and elucidation of mechanisms regulating these divergent pathways is of obvious importance for developing effective clinical treatments. Most studies of pathogenic TNFR1-dependent signaling have focused on changes in the expression level of TNFRI or associated signaling molecules (21–23) whereas limited attention has been paid to potential mechanisms that control TNFR1 activity independently of variant protein expression, for example, regulation via post-translational modifications such as glycosylation. To date there is very little information concerning the glycosylation of TNFR1. Analyses of the secreted form of TNFR1 present in human urine suggest that TNFR1 is elabo-

FIGURE 6. ST6Gal-I activity causes increased α2-6 sialylation of TNFR1 in vivo. A, construct used to generate ST6Gal-I-expressing transgenic mice. B, splenic homogenates were prepared from wild-type (WT) or transgenic mice, and immunoblotting was performed with anti-V5 antibody and anti-ST6Gal-I antibody. C, peritoneal cells collected 96 h following thioglycollate injection were immunoblotted for ST6Gal-I. D, α2-6 sialylated proteins from peritoneal cell lysates were precipitated with SNA-agarose and immunoblotted for TNFR1. E, cell lysates from wild-type and transgenic mice were immunoblotted for TNFR1.

FIGURE 7. ST6Gal-I inhibits apoptosis of macrophages from ST6Gal-I transgenic mice. A, peritoneal cells were harvested at 96 h following thioglycollate injection and nucleated cell counts were obtained using a hemacytometer (n = 10 for each group of animals). B, peritoneal cells were stained with annexin V and PI, and the apoptosis rate was analyzed by flow cytometry (n = 11 WT; n = 10 line 1; n = 9 line 2). C and D, peritoneal cells were cultured overnight, and the adherent fraction (representing macrophages) was treated with TNFα plus CHX for 24 h. Annexin V and PI staining revealed significantly lower levels of apoptosis in each of the two transgenic lines. (For panel C, n = 5 for each group; for panel D, n = 8 for each group.)

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Sialylation of TNFR1 inhibits apoptosis, as demonstrated in the current study. This inhibition is mediated by the enzyme ST6Gal-I, which plays a critical role in the glycosylation of TNFR1. The results presented in this study suggest that regulating the expression and activity of ST6Gal-I could have therapeutic implications for the treatment of autoimmune disorders. The findings also highlight the importance of glycosylation in the regulation of death receptor function and the potential for targeting these pathways in the development of novel therapeutic strategies. Further studies are needed to elucidate the molecular mechanisms underlying the observed effects and to assess the clinical relevance of these findings.
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