Multiple sclerosis (MS) is characterized by inflammatory demyelination of axons and neurodegeneration, the latter inadequately modeled in experimental autoimmune encephalomyelitis (EAE). Susceptibility of inbred mouse strains to EAE is in part determined by major histocompatibility complex haplotype; however, other molecular mechanisms remain elusive. Galectins bind GlcNAc-branched N-glycans attached to surface glycoproteins, forming a molecular lattice that restricts lateral movement and endocytosis of glycoproteins. GlcNAc branching negatively regulates T cell activity and autoimmunity, and when movement and endocytosis of glycoproteins. GlcNAc branching negatively regulates T cell activity and autoimmunity, and when absent in neurons, induces apoptosis in vivo in young adult mice. We find that EAE susceptible mouse strains PL/J, SJL, and NOD have reduced GlcNAc branching. PL/J mice display the lowest levels, partial deficiencies in N-acetylgalcosaminyltransferase I, II, and V (i.e., Mgat1, -2, and -5), T cell hyperactivity and spontaneous late onset inflammatory demyelination and neurodegeneration; phenotypes markedly enhanced by Mgat5+/− and Mgat5−/− backgrounds in a gene dose-dependent manner. Spontaneous disease is transferable and characterized by progressive paralysis, tremor, dystonia, neuronophagia, and axonal damage in both demyelinated lesions and normal white matter, phenocopying progressive MS. Our data identify hypomorphic Golgi processing as an inherited trait that determines susceptibility to EAE, provides a unique spontaneous model of MS, and suggests GlcNAc-branched deficiency may promote T cell-mediated demyelination and neurodegeneration in MS.

Relapsing remitting multiple sclerosis is characterized by inflammatory destruction of the myelin sheath surrounding axons in the central nervous system (CNS), producing relapsing remitting attacks of neurological dysfunction (1). This is commonly followed by a secondary progressive neurodegenerative phase distinguished by axonal damage and neuronal loss (1). Primary progressive MS is similar to secondary progressive disease but lacks the initial relapsing remitting phase. However, recent investigations have demonstrated that gray matter involvement and axonal damage in otherwise normal appearing white matter are present at the onset of relapsing remitting multiple sclerosis (2, 3). This indicates neurodegeneration is an early and prominent feature of disease and questions the interpretation that MS is only a T cell-mediated demyelinating disease. Experimental autoimmune encephalomyelitis (EAE) is a useful model of T cell-dependent inflammatory demyelination, but fails to properly address the neurodegenerative phenotype of MS.

MS is characterized by adult onset and partially familial relationships, indicating complex interactions between environmental and genetic factors in disease pathogenesis (4). Whole genome screens have identified a number of candidate loci associated with MS (5) and EAE (6, 7), but non-MHC genes that strongly promote disease have yet to be described. This is despite long standing observations that T cell dysfunction is critical to development of EAE (8–10) and the identification of multiple genes that alter EAE severity in susceptible mouse strains when deficient or overexpressed (11). Myelin-specific TCR transgenic mice develop spontaneous CNS autoimmune demyelinating disease in susceptible strains (9, 10, 13, 14), however, spontaneous disease secondary to physiologically relevant gene dysfunction has not been reported.

Deficiency of the N-glycan processing gene Mgat5 in 129/Sv mice, an EAE-resistant strain, results in spontaneous kidney autoimmunity after 1 year of age, enhanced delayed type hypersensitivity, and increased susceptibility to myelin basic protein (MBP)-induced EAE (15). Mgat5 is near the end of a linear pathway of Golgi processing enzymes required for GlcNAc-branching in N-glycans, structures on glycoproteins that serve as ligands for the galectin family of N-acetyllactosamine binding lectins. Multivalent binding between N-glycans attached to surface glycoproteins and galectins forms a molecular lattice that restricts lateral movement of glycoproteins and their loss to endocytosis (15, 16). Galectin binding to N-glycans increases proportionally for mono-, bi-, tri-, and tetra-antennary GlcNAc-branched N-glycans, the products of N-acetylgalcosami-
spontaneous diabetes in non-obese diabetic (NOD) mice (21). These data demonstrate that metabolism, via UDP-GlcNAc biosynthesis, conditionally regulates T cell-mediated autoimmunity by altering GlcNAc branching in N-glycans.

Here we report that among inbred mouse strains, N-glycan GlcNAc-branched in T cells is highly variable and inversely correlates with EAE susceptibility. PL/J mice display the lowest levels, partial deficiency of Mgtat1, -2, and -5 enzyme activity, TCR hypersensitivity, and mild spontaneous inflammatory demyelination and neurodegeneration after 1 year of age. Spontaneous disease was markedly enhanced by Mgtat5+/− and Mgtat5−/− backgrounds in a gene dose-dependent manner, demonstrating interactions between inherited and experimentally induced defects in N-glycan processing. PL/J mice with spontaneous disease displayed features of chronic MS, including progressive paralysis, tremor, focal dystonic posturing, paroxysmal dystonia, neuronophagia, and axonal damage in demyelinated lesions and normal white matter (1, 25). Our results indicate that naturally arising hypomorphisms in multiple N-glycan GlcNAc-branched enzymes regulate EAE susceptibility among inbred strains of mice. Moreover, Mgtat5−/− PL/J mice provide a unique spontaneous model of MS that arises from physiologically relevant gene dysfunction and displays the two critical phenotypes observed in MS, namely inflammatory demyelination and neurodegeneration.

EXPERIMENTAL PROCEDURES

FACS Analysis and in Vitro Proliferation Assays—The PL/J and C57BL/6 mice were congenic at backcross 6 from 129/Sv and showed no difference in L-PHA staining compared with PL/J and C57BL/6 mice obtained from Jackson Laboratories. 129/Sv mice were from our original Mgtat5 gene targeted population. All other mice (SJL, NOD, Balb/c, and B10.S) were obtained from Jackson Laboratories and acclimatized prior to use. Mice used for FACS staining and proliferation assays were sex and age matched and housed in the same cage. All procedures and protocols with mice were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Mouse cells were stained with anti-CD4 (RM4–5), anti-CD8 (53-6.7), anti-CD45R (RA3–6B2), anti-CD25 (PC61), anti-CD69 (H1.2F3), anti-CD62L (MEL-14), anti-TIM-3 (8B.2C12), anti-Foxp3 (FKJ-16s), anti-CD11b (M1/70), and anti-F4/80 (BM8) from e Bioscience and Phaseolus vulgaris leukoagglutinating lectin (L-PHA, 4 µg/ml) from Vector Laboratories. Purified CD3+ T cells (R&D Systems) were labeled with 5 µM 5,6-carboxyfluorescein diacetate succinimidy l ester (CFSE; Molecular Probes) in phosphate-buffered saline for 8 min at room temperature and stimulated with plate-bound anti-CD3ε (2C11, e Bioscience) in the presence or absence of swainsonine (SW) (Sigma).

TCR Signaling—TCR signaling was preformed as previously described (21). The following antibodies were used: hamster anti-CD3e (2C11, e Bioscience), rabbit anti-phospho-Src family Tyr416 (Cell Signaling Technology), which cross-reacts with phospho-lck Tyr394, rabbit anti-phospho-LAT (Upstate), and anti-actin (Santa Cruz).

Enzymatic Assays—Enzyme activity was measured using synthetic specific acceptors. The acceptors for Mgtat5 (GnTV), Mgtat2 (GnTIII), and Mgtat1 (GnTII) were βGlcNAc(1,2)αMan(1,6) βGlc-O(CH2)7CH3, βGlcNAc(1,2)αMan(1,3)αMan(1,6)βMan-O(CH2)7CH3, αMan(1,3)βMan-O(CH2)7CH3, respectively (Toronto Research Chemicals). 10 µl of cell lysate (0.9% NaCl, 1% Triton X-100 on ice, centrifuged 5000 × g for 15 min at 4 °C) was added to 1 mM acceptor, 1 mM [6-3H]UDP-GlcNAc (American Biosciences) in 50 mM MES, pH 6.5, 0.1 mM GlcNAc, and 25 mM AMP for a total reaction volume of 20 µl. Mgtat2 and Mgtat1 reactions also contained 5 mM MnCl2 and were incubated for 1 h; Mgtat5 for 3 h at 37 °C. Reaction was stopped with 1 ml of ice-cold water. Enzyme products were separated from radioactive substrates by binding to 50-mg C18 cartridges (Alltech) preconditioned with methanol rinsing and water washing. Reactions were loaded and the columns washed 5 times with 1 ml of water. Radiolabeled products were eluted directly into scintillation vials with two separately applied 0.5-ml aliquots of methanol and the radioactivity was determined by liquid scintillation counting.

Quantitative Real-time PCR—RNA from purified CD3+ T lymphocytes of 129/sv, PL/J, and C57BL/6 mice was purified using the RNeasy® Mini Kit (Qiagen) and used to synthesize cDNA with the RETROscript® Kit (Ambion). For expression of mouse Mgtat1, -2, and -5 and β-actin, a 7900HT platform (3840-well plate, Applied Biosystems) was used with SYBR® Green PCR master mixture and the following primers: Mgtat5,
Spontaneous Mouse Model of Multiple Sclerosis

EAE Susceptible Mouse Strains Are Hypomorphic for N-Glycan GlcNAc Branching—Reducing GlcNAc-branching by $\pm 20–25\%$, either by loss of a single Mgat5 allele or by partial inhibition of Golgi processing with the mannosidase II inhibitor SW, is sufficient to enhance TCR signaling and T cell proliferation (Ref. 21 and Fig. 1A and supplemental Fig. S1). Therefore, we explored whether susceptibility of inbred mouse strains to EAE correlates with levels of GlcNAc-branching. For this purpose we stained the cell surface with L-PHA (P. vulgaris leukoagglutinin), a plant lectin that specifically binds $\beta_1,6$-GlcNAc-branched N-glycans produced by Mgat5 and serves as a marker of GlcNAc-branching (15, 21). CD4$^+$ and CD8$^+$ T cells from the EAE susceptible strains PL/J, SJL, and NOD, which also develops spontaneous autoimmune diabetes, expressed $\sim 30–40\%$ less $\beta_1,6$GlcNAc-branched N-glycans than the three EAE-resistant strains 129/Sv, Balb/c, and B10.S (Fig. 1, A–C). Remarkably, CD4$^+$ T cells from wild-type PL/J mice express $\sim 25\%$ less $\beta_1,6$GlcNAc-branched N-glycans than Mgat5 heterozygous 129/Sv cells (Fig. 1A). This indicates that the PL/J strain harbors genetic hypomorphisms that reduce GlcNAc-branching to a significantly greater degree than loss of an Mgat5 allele. The C57BL/6 strain is less sensitive than the SJL strain to EAE, as evidenced by differential requirement for CD28 co-stimulation to induce disease (26). C57BL/6 T cells display intermediate levels of $\beta_1,6$GlcNAc-branched N-glycans relative to T cells from the EAE-sensitive and -resistant strains (Fig. 1, B and C). Co-staining CD4$^+$ T cells with L-PHA and the naive T cell marker CD62L, the effector/regulatory T cell marker CD25, and/or the regulatory T cell marker Foxp3 demonstrated the same relative differences in $\beta_1,6$GlcNAc-branched N-glycans, with PL/J $<$ C57BL/6 $<$ 129/Sv (Fig. 1D). However, in B220$^+$ B cells and F4/80$^+$ CD11b$^+$ macrophages, $\beta_1,6$GlcNAc-branched N-glycan levels were similar among the tested strains (Fig. 1, B and C). Therefore, susceptibility to EAE correlated inversely with $\beta_1,6$GlcNAc-branched N-glycan expression in T cells in rank order PL/J $>$ SJL, NOD $>$ C57BL/6 $>$ Balb/c, 129/Sv, B10.S.

RESULTS

Electromyography and Nerve Conduction Studies—Mice were anesthetized with Avertin. Temperature was maintained at 35–37 °C using infrared heat lamps. Monopolar needle electrodes (Ambu Inc., Glen Burnie, MD) were used for stimulation and recording motor nerve potentials. The active and indifferent recording electrodes were placed in medial gastrocnemius and ipsilateral footpad, respectively. The active and reference stimulating electrodes were placed percutaneously in the popliteal or sciatic notch and ipsilateral thoraco-lumbar paraspinal muscle, respectively. A pre-gelled strip electrode at the tail acted as a ground. Responses from supramaximal electrical stimulation (pulse width 0.05 ms) were analyzed with additional stimulations done to record late responses (F waves and H reflexes). H reflexes were identified when successive late responses had identical morphology and onset latency; F waves were identified when successive late responses had variable onset latency and morphology. For needle EMG recording, the recording monopolar needle electrode was inserted into one or more hindlimb muscles: quadriceps, hamstrings, lumbar paraspinals, gastrocnemius, and tibialis anterior. The presence of spontaneous muscle activity (i.e. fasciculations, fibrillations, or myokymia) was assessed in at least three regions of the muscle. All recordings were made on a Sierra LT portable machine (Cadwell Laboratories, Kennewick, WA) and analyzed using the proprietary software supplied by the manufacturer.
Next we explored potential mechanisms for reduced β1,6GlcNAc-branching in PL/J T cells. The final step in the linear pathway to β1,6GlcNAc-branched N-glycan biosynthesis is mediated by Mgat5. Mgat5 enzyme activity but not mRNA levels are reduced ~50% in splenocytes and T cells from PL/J and C57BL/6 mice relative to 129/Sv mice (Fig. 2, A and B).
However, PL/J T cells display a greater reduction in β1,6GlcNAc-branched N-glycans than C57BL/6 T cells, suggesting additional N-glycan processing defects proximal to Mgat5 are present in PL/J cells. Indeed, MALDI-TOF mass spectrometry indicates that relative to 129/Sv and C57BL/6 T cells, PL/J T cells have reduced bi- and triantennary GlcNAc-branched N-glycans (E ions) and accumulate pathway intermediates upstream of Mgat2 (C and D ions) (Fig. 2C and supplemental Fig. S2). Moreover, Mgat2 and Mgat1 enzymatic activities, but not mRNA transcription levels, differ significantly among the three strains, with PL/J < C57BL/6 < 129/Sv and PL/J, C57BL/6 < 129/Sv, respectively (Fig. 2, A and B). These data indicate that partial deficiencies at the post-transcriptional level in Mgat1, -2, and -5 combine to reduce GlcNAc-branched in PL/J > C57BL/6 > 129/Sv T cells and confirm that PL/J mice are naturally hypomorphic for GlcNAc-branched N-glycans. However, defects in other N-glycan processing enzymes may also contribute to the phenotype.

Deficiency of Multiple Golgi GlcNAc Transferases Induces T Cell Hyperactivity in PL/J Mice—

Mgat5+/− 129/Sv T cells hyperproliferate relative to Mgat5+/+ 129/Sv T cells (supplemental Fig. S1); indicating the greater reduction in GlcNAc-branched inherent to wild-type PL/J T cells should result in T cell hyperactivity. Indeed, wild-type PL/J T cells were more sensitive to TCR agonist than wild-type 129/Sv T cells as indicated by phosphorylation levels of lck at activating Tyr394 and LAT, induction of the activation marker CD69, and proliferation as measured by CFSE dilution (Fig. 3, A–D). Consistent with intrinsically higher β1,6GlcNAc-branched in the 129/Sv strain, the relative increase in lck-pY394, pLAT394, and CD69 is significantly greater in Mgat5−/− versus Mgat5+/+ 129/Sv T cells than Mgat5+/− versus Mgat5+/+ PL/J T cells (Fig. 3, A and B). However, in absolute terms, TCR signaling and CD69 expression were greater in PL/J Mgat5−/− than 129/Sv Mgat5−/− T cells (Fig. 3, A and B). Galectins bind N-glycans in proportion to GlcNAc-branched (17, 18) and these data suggest that deficiency of bi- and triantennary GlcNAc-branched N-glycans also contribute to PL/J T cell hypersensitivity. Indeed, co-incubation of wild-type 129/Sv cells with SW, which blocks N-glycan GlcNAc-branched beyond monoantennary, equalized L-PHA staining, CD69 expression, and proliferation to that of untreated wild-type PL/J T cells (Fig. 3, C and D, supplemental Figs. S3 and S4). In contrast, SW had nominal affects on wild-type PL/J T cells, consistent with pre-existing deficiency in bi- and triantennary structures. SW also had minimal affects on Mgat5−/− PL/J T cells but enhanced CD69 expression in Mgat5−/− 129/Sv T cells to that of Mgat5−/− PL/J T cells co-incubated with or without SW (Fig. 3E). Together, these data indicate that GlcNAc-branched deficiency in wild-type PL/J T cells induces TCR hyperactivity relative to 129/Sv, with reductions in bi-, tri-, and tetra-antennary GlcNAc-branched N-glycans all contributing to the phenotype.

The above data suggest that genetic rescue of GlcNAc-branched deficiency in PL/J T cells will require enhancing the expression of at least three genes (i.e. Mgat1, -2, and -5). In contrast, metabolically supplementing the hexosamine pathway with GlcNAc increases bi-, tri-, and tetra-antennary GlcNAc-branched N-glycans by increasing UDP-GlcNAc supply.
GlcNAc-branching deficiency in PL/J mice induces T cell hyperactivity. A, purified CD3⁺ T cells from PL/J and 129/Sv mice were incubated at 37 °C with anti-CD3 antibody-coated beads for various times, lysed, and Western blotted (WB). Bands quantified using Gel Pro Analyzer software were normalized initially to actin (WB: Actin) and then to actin. Numbers are shown below each band. B–E, purified CD3⁺ T cells from PL/J and 129/Sv mice of the indicated genotypes unlabeled (B, C, and D) or labeled with CFSE (D) were stimulated with anti-CD3 antibody-coated beads for various times, and analyzed by FACS. All data are gated on CD4⁺ cells. Error bars represent mean ± S.E. of triplicate values. Results are representative of at least three independent experiments.

FIGURE 3. GlcNAc-branched deficiency in PL/J mice induces T cell hyperactivity. A, purified CD3⁺ T cells from PL/J and 129/Sv mice were incubated at 37 °C with anti-CD3 antibody-coated beads for various times, lysed, and Western blotted (WB). Bands quantified using Gel Pro Analyzer software were normalized initially to actin (WB: Actin) and then to actin. Numbers are shown below each band. B–E, purified CD3⁺ T cells from PL/J and 129/Sv mice of the indicated genotypes unlabeled (B, C, and D) or labeled with CFSE (D) were stimulated with anti-CD3 antibody for 48 h, stained with CD4 and CD69, and analyzed by FACS. All data are gated on CD4⁺ cells. Error bars represent mean ± S.E. of triplicate values. Results are representative of at least three independent experiments.

to Mgat1-/-, -2, and -5 (17, 21, 23), providing a simple experimental approach for rescue. Indeed, supplementing PL/J T cells with GlcNAc rescues N-glycan GlcNAc-branched and inhibits TCR signaling, CD69 expression, T₄₁ differentiation, CTLA-4 endocytosis, and proliferation (21). These inhibitory phenotypes are all reversed by co-incubation with SW, confirming that GlcNAc supplementation acts by increasing GlcNAc-branched in N-glycans. Taken together, these data demonstrate that hypomorphic production of GlcNAc-branched N-glycans in PL/J mice is causal in T cell hypersensitivity.

Defective N-Glycan Processing in PL/J Mice Promotes Spontaneous Inflammatory Demyelination and Neurodegeneration—Mgat5⁻/⁻/⁻ 129/Sv mice develop spontaneous kidney autoimmunity after 1 year of age and are more susceptible to EAE (15). Neuron-specific depletion of the Mgat1 gene eliminates galectin ligands in N-glycans and results in neuronal apoptosis in young adult mice (27). High levels of galectin-1, which provokes T cell apoptosis by remodeling the galectin lattice (28, 29), induces axonal and neuronal cell body degeneration (30). In addition to N-glycan processing deficiency, the PL/J strain possess the H-2b⁺ MHC class II haplotype that reduces negative selection of MBP 1–11 reactive T cells in the thymus (31, 32). We reasoned that loss of central tolerance to MBP1–11 combined with reduced N-glycan GlcNAc-branching may induce spontaneous CNS inflammatory demyelination and neurodegeneration in PL/J mice. Indeed, clinical observation of Mgat5⁺/+⁺, Mgat5⁻/+⁺, and Mgat5⁻⁻/⁻ PL/J mice at backcross 4 and 6 from 129/Sv as well as non-congenic wild-type PL/J mice from Jackson Laboratories revealed signs of tail and/or hindlimb weakness after 1 year of age (Table 1, supplemental Table 1, supplemental Video 1, and data not shown). Importantly, incidence, severity, and mortality were inversely correlated with Mgat5 gene dose (Table 1 and supplemental Table 1), demonstrating genetic interactions of inherited and experimentally induced deficiencies in GlcNAc-branched N-glycans. The course was chronic and slowly progressive without relapses or recovery (supplemental Fig. 5F), a clinical picture typical of progressive MS (1). Mice frequently displayed involuntary movements such as tremor, focal dystonic posturing, and paroxysmal dystonia (Fig. 4A, supplemental Video 1, B–D, and Table 1), movement disorders that occur in MS (25) but rarely reported in EAE.

Pathological studies revealed submeningeal perivascular lymphocyte cuffing (Fig. 4B) and multifocal demyelination of the brainstem (Fig. 4, C and D), spinal cord (supplemental Fig. S5, A and B) and spinal roots (Fig. 4, G and H, supplemental Fig. S5, C and E). CNS pathology was similar to chronic MS plaques and characterized by mononuclear cells admixed with myelin debris centered around blood vessels, gliosis, axonal swelling (spheroids), and axonal degeneration. Phagocytosis of neurons in the gray matter (neuronophagia, Fig. 4E), a presumptive marker of neuronal apoptosis, and axonal pathology in otherwise normal appearing CNS white matter (Fig. 4F) were also frequently observed; phenotypes consistent with previous data demonstrating a direct affect of GlcNAc-branched on neurodegeneration (27). Gray matter disease and axonal damage in normal appearing white matter are early and diffuse features of

- The image contains a page from a scientific journal article discussing the spontaneous mouse model of multiple sclerosis (MS) and the role of the Mgat5 gene in this model. The text describes experimental results showing that Mgat5 deficiency in PL/J mice induces T cell hyperactivity and contributes to spontaneous CNS inflammatory demyelination and neurodegeneration. The article highlights the importance of N-glycan processing in the pathogenesis of MS.

- The text includes a figure (FIGURE 3) illustrating the results of T cell proliferation assays under different conditions, with data showing the effects of Mgat5 deficiency on T cell responses.

- The article discusses the implications of these findings for understanding the genetic and molecular basis of MS and other related neurological disorders.
TABLE 1
Clinical observations of spontaneous disease in PL/J mice
Severity of weakness was scored on a scale of 0–5 with: 0, no weakness; 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness/paralysis and hindlimb paralysis; and 5, moribund or death. Severity of dystonia was scored on a scale of 0–3 with: 0, no dystonia; 1, tail dystonia; 2, hindlimb dystonia; 3, axial skeleton and/or paroxysmal dystonia. Mice were housed at the Samuel Lunenfeld Research Institute animal colony containing mouse hepatitis virus, EDIM, minute virus, mouse parvovirus, GDVII, pinworm, and fur mites. Time and age are given in months. Percentages for spontaneous death and incidence were cumulative. Severity is mean ± S.E. n was the denominator for all calculations.

| Genotype       | n  | Age    | Weakness | Dystonia | Death |
|----------------|----|--------|----------|----------|-------|
|                |    | months |          |          |       |
| Mgat5+/+       | 10 | 14 ± 0.4 | 20 | 0.4 ± 0.27 | 0 |
| Mgat5−/−       | 13 | 15.6 ± 0.7 | 38.5 | 0.8 ± 0.28 | 23.1 |
| Mgat5−/+       | 21 | 18.2 ± 0.8 | 69.2 | 1.6 ± 0.38 | 38.5 |

Adoptive transfer of demyelinating disease into wild-type PL/J mice
Spontaneously diseased Mgat5−/− (n = 4) and Mgat5−/+ (n = 6) donor mice were scored for severity of demyelinating pathology (+ to +++) and 1 × 10^6 splenocytes were injected intraperitoneally into naive wild-type PL/J mice following 48 h of in vitro activation with anti-CD3/anti-CD28.Recipient mice were scored for weakness over a 2-month period and presence of demyelinating pathology (mean ± S.E.).

| Donor Genotype | Age | Severity | Incidence | Onset Score | Pathology |
|---------------|-----|----------|-----------|-------------|-----------|
| Mgat5+/+      | 18 ± 3 | ++/++/++ | 1/6 | 3.03 ± 0.33 | 1/4 |
| Mgat5−/−      | 13 ± 2 | ++ | 0/6 | 0 | 0/6 |
| Mgat5−/+      | 19 ± 1 | ++/++/++ | 3/3 | 3 ± 1.3 | 3/3 |

MS (1–3) that are generally lacking in typical EAE. Pathology in the peripheral nervous system was characterized by multifocal spinal root demyelination with naked and swollen axons (Fig. 4H, supplemental Fig. SSE). Neuronal bodies with prominent central chromatolysis were observed in the spinal cord (supplemental Fig. 5D), consistent with anterograde reaction to peripheral axonal damage. Electromyography and nerve conduction studies confirmed physiological spinal root demyelination and axonal damage, revealing myokymia, positive sharp waves (supplemental Fig. 5G), and delayed spinal root nerve conduction velocity as evidenced by abnormal F and H responses (supplemental Fig. 5H).

CNS and/or peripheral nerve system pathology was present and qualitatively similar in all mice with clinical weakness, and frequently co-existed in the same individual. Additional organ screening in 4 clinically affected mice suggested that the only autoimmune disease present was inflammatory demyelination. Peripheral nervous system demyelination was seen with similar frequency in all three Mgat5 genotypes (Fig. 4I). In contrast, CNS disease was ~2- and 3-fold more frequent in Mgat5−/− and Mgat5−/+ PL/J mice than wild-type mice, respectively.

Anti-CD3 antibody stimulated splenocytes from Mgat5−/− mice with moderate to severe, but not mild demyelinating pathology, frequently transferred disease to naive wild-type recipients (Table 2), confirming that spontaneous disease was in part immune mediated. Metabolic supplementation (i.e. GlcNAC) enhances GlcNAC-branding in encephalitogenic wild-type PL/J T cells in vitro and inhibits their ability to induce EAE.
when the cells are transferred to naïve recipients (21). This confirms that reduced GlcNAc-branching in PL/J T cells promotes T cell hypersensitivity and demyelinating disease in vivo.

Environmental pathogens have been implicated in demyelinating disease in MBP-TCR transgenic mice (9). However, we observed similar frequency of disease when mice were housed in vivariums containing a multitude of pathogens (Table 1), a single pathogen or no pathogens (supplemental Table 1), suggesting genetic rather than infectious factors dominate in disease pathogenesis.

\textit{Mgat5}\textsuperscript{−/−} PL/J mice <1 year and diseased PL/J mice of all three genotypes >1 year displayed increased frequency of TIM-3\textsuperscript{+} T\textsubscript{h}1 cells (Fig. 5A), indicating negative regulation of TIM-3\textsuperscript{+} T\textsubscript{h}1 differentiation by GlcNAc-branching in vivo (19). Paradoxically, the \textit{Mgat5} deficiency, increasing age, and demyelinating disease are associated with increased numbers of CD25\textsuperscript{+}CD4\textsuperscript{+} and CD25\textsuperscript{+}Foxp3\textsuperscript{+}CD4\textsuperscript{+} T cells (Fig. 5B, supplemental Fig. S6). This result is consistent with hyperproliferation of \textit{Mgat5}\textsuperscript{−/−} regulatory T cells observed in vitro\textsuperscript{4} and previous observations of age-associated increases in functional CD25\textsuperscript{+}CD4\textsuperscript{+} regulatory T cells in humans (33). The increased frequency of PL/J CD25\textsuperscript{+}Foxp3\textsuperscript{+}CD4\textsuperscript{+} regulatory T cells in vivo may not only be a direct effect of GlcNAc-branching deficiency on proliferation, but also reflect homeostatic negative regulation of autoreactive T\textsubscript{h}1 effector cells. Taken together these data indicate enhanced T\textsubscript{h}1 effector responses associated with the GlcNAc-branching deficiency combined with H-2\textsuperscript{a}

\textit{DISCUSSION}

GlcNAc-branching in N-glycans attached to surface glycoproteins promotes multivalent galectin binding, which negatively regulates TCR signal strength and T\textsubscript{h}1 differentiation of naïve T cells, CTLA-4 endocytosis in activated T cells, and susceptibility to autoimmunity (15, 19). Here we demonstrate that variability in T cell-specific expression of GlcNAc-branching N-glycans is an inherited trait in inbred mice that regulates T cell function and susceptibility to autoimmune demyelinating disease. EAE susceptible strains PL/J, NOD, and SJL are hypomorphic for GlcNAc-branching N-glycans in T cells but not B cells and macrophages; a phenotype that induces T cell hyperactivity and promotes EAE susceptibility in PL/J mice. A mild inflammatory demyelinating disease similar to progressive MS develops spontaneously in PL/J mice after 1 year of age, a phenotype markedly enhanced by gene dose-dependant loss of \textit{Mgat5}. This demonstrates inherited and induced defects in GlcNAc-branching and disease at the genetic level to promote disease. However, our data does not exclude other molecular mechanisms in the development of spontaneous disease.

Loss of β1,6GlcNAc-branching N-glycans via genetic deletion of \textit{Mgat5} enhances demyelinating disease in both 129/Sv H-2\textsuperscript{b} (15) and PL/J H-2\textsuperscript{a} mice. \textit{Mgat5}\textsuperscript{−/−} 129/Sv mice develop late onset spontaneous kidney autoimmunity, but unlike the PL/J strain, \textit{Mgat5}\textsuperscript{−/+} and \textit{Mgat5}\textsuperscript{−/−} 129/Sv mice do not develop spontaneous autoimmune disease; a result consistent with the higher levels of GlcNAc-branching N-glycans in the 129/Sv strain (15). These data suggest GlcNAc-branching regulates autoimmune thresholds and EAE irrespective of the MHC haplotype, whereas strain-dependent genetic factors such as MHC determine the targeted tissue in spontaneous disease.

Autoimmunity is a complex trait, where genetic susceptibility is distributed across multiple genes and modulated by the environment via unclear mechanisms. Genetic and environmental factors are often presumed to encompass various pathways that interact by unknown molecular mechanisms. Remarkably, we find that reduced GlcNAc-branching in PL/J T cells results from partial deficiencies of multiple N-glycan processing enzymes, including Mgat1, -2, and -5, but can be conditioned regulated by metabolite input to the biosynthesis of UDP-GlcNAc (21). This indicates a unique genetic model for autoimmunity, whereby multiple weakly penetrant genetic factors combine in the Golgi N-glycan GlcNAc-branching pathway to produce a highly penetrant phenotypic change to promote disease. This model raises the possibility that co-inheritance of genetic variants in multiple N-glycan pathway genes may combine to reduce GlcNAc branching and promote MS in humans.

MS is frequently a two-stage disease characterized by inflammatory destruction of the myelin sheath with mean onset ~29 years of age followed ~10 years later by a secondary progressive neurodegenerative phase distinguished by axonal damage and neuronal loss (1, 34). The spontaneous demyelinating disease in PL/J mice phenocopies several important clinical features of

\textsuperscript{4} S.-U. Lee and M. Demetriou, unpublished data.
progressive MS: spontaneous onset in mid-life, movement disorders such as tremor and dystonia, and a slow progressive decline in neurological function in association with neuronal loss and axonal damage (1, 25). As such, Mgat5-deficient PL/J mice provide a unique model to study both the inflammatory and neurodegenerative components of MS. Neuron-specific loss of Mgat1, which eliminates galectin ligands in N-glycans, results in apoptosis of adult neurons in vivo (27). This suggests that defects in GlcNAc branching inherent to the PL/J strain, coupled with induced deficiency in Mgat5, may promote neurodegeneration in Mgat5−/− PL/J mice independent from effects on T cell function. Additional investigation is required to confirm this hypothesis. Moreover, it will be important to determine the relative roles of T cell-mediated demyelination and neurodegeneration. Supplementing the hexosamine pathway with GlcNAc increases GlcNAc-branched in multiple cell types (17, 21, 23), raising the possibility that in addition to suppressing inflammatory demyelination (21), this therapeutic approach may also directly limit neurodegeneration in MS.

NOD and SJL mice also display reduced β1,6GlcNAc-branched in T cells, albeit less severe than the PL/J strain. Oral GlcNAc supplementation increases N-glycan GlcNAc branching in vivo and suppresses spontaneous autoimmune diabetes in NOD mice (21), indicating GlcNAc branching deficiency also promotes spontaneous autoimmunity in the NOD strain. Although NOD mice harbor the H-2Kd MHC haplotype, a critical promotor of diabetes, they also develop other spontaneous autoimmune diseases at lower frequency (e.g. sialitis, autoimmune thyroiditis) (35) and when given pertussis toxin, autoimmune diabetes is suppressed and spontaneous CNS demyelinating disease develops (36). Similarly, B7-2 deficiency or interleukin-2 blockade in NOD mice induces spontaneous peripheral nerve autoimmune demyelination (37, 38). Schwann cells, the cellular constituent of peripheral myelin, surround pancreatic islets and have been proposed to be targeted early in the development of insulitis in NOD mice (39). SJL mice are highly sensitive to EAE, in part because of high precursor frequency of proteolipid protein 139–151 reactive T cells (40); however, spontaneous autoimmunity has not been reported in this strain. The level of N-glycan GlcNAc branching appears similar in NOD and SJL T cells, suggesting hypomorphic expression of GlcNAc-branched N-glycans in these mice promote T cell-mediated demyelinating disease, but other genetic factors are required for induction of spontaneous disease.

Modification of β1 integrins with β1,6GlcNAc-branched N-glycans reduces cell adhesion to fibronectin and increases cell motility (16, 24, 41, 42), phenotypes that may contribute to T cell dysfunction and disease. Antibodies to α5β1 integrin inhibit EAE and MS by limiting T cell recruitment to the CNS via interactions with vascular cell adhesion molecule-1 expressed on activated endothelium (43, 44). β1,6GlcNAc branching reduces T cell adhesion to vascular cell adhesion molecule in vitro,5 suggesting β1,6GlcNAc-branched may limit T cell recruitment to the CNS in vivo. Macrophage motility and phagocytosis are impaired by Mgat5 deficiency (16), a phenotype that may inhibit clearance of apoptotic cells and promote autoimmunity (12). However, macrophages from wild-type PL/J, C57BL/6, and 129/Sv mice display similar levels of β1,6GlcNAc-branched N-glycans, suggesting defective N-glycan processing does not significantly alter macrophage function to promote autoimmunity in wild-type PL/J mice. Defects in substrate and/or cell-cell adhesion may also occur in neurons and directly contribute to the neurodegeneration observed in Mgat5−/− PL/J mice. With greater insight into the relative importance of GlcNAc branching in these various pathways, we will achieve a more complete molecular model of spontaneous demyelinating disease and neurodegeneration.

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