THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 27, pp. 20027–20035, July 6, 2007

Control of T Cell-mediated Autoimmunity by Metabolite Flux to *N*-Glycan Biosynthesis*[S]

Received for publication, March 5, 2007, and in revised form, April 19, 2007 Published, JBC Papers in Press, May 8, 2007, DOI 10.1074/jbc.M701890200

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Autoimmunity is a complex trait disease where the environment influences susceptibility to disease by unclear mechanisms. T cell receptor clustering and signaling at the immune synapse, T cell proliferation, CTLA-4 endocytosis, T_H1 differentiation, and autoimmunity are negatively regulated by β1,6GlcNAc-branched *N*-glycans attached to cell surface glycoproteins. β1,6GlcNAc-branched *N*-glycan expression in T cells is dependent on metabolite supply to UDP-GlcNAc biosynthesis (hexosamine pathway) and in turn to Golgi *N*-acetylglucosaminyltransferases Mgat1, -2, -4, and -5. In Jurkat T cells, β1,6GlcNAc-branching in *N*-glycans is stimulated by metabolites supplying the hexosamine pathway including glucose, GlcNAc, acetoacetate, glutamine, ammonia, or uridine but not by control metabolites mannosamine, galactose, mannose, succinate, or pyruvate. Hexosamine supplementation *in vitro* and *in vivo* also increases β1,6GlcNAc-branched *N*-glycans in naïve mouse T cells and suppresses T cell receptor signaling, T cell proliferation, CTLA-4 endocytosis, T_H1 differentiation, experimental autoimmune encephalomyelitis, and autoimmune diabetes in non-obese diabetic mice. Our results indicate that metabolite flux through the hexosamine and *N*-glycan pathways conditionally regulates autoimmunity by modulating multiple T cell functionalities downstream of β1,6GlcNAc-branched *N*-glycans. This suggests metabolic therapy as a potential treatment for autoimmune disease.

Complex trait diseases such as autoimmunity are determined by poorly understood genetic and environmental interactions. The T cell-mediated autoimmune diseases multiple sclerosis (MS)[3] and type 1 diabetes exemplify this problem, where identical twins of Northern European descent are discordant ~60–70% of the time despite displaying an ~150–300 times higher risk than the general population prevalence of ~0.1 and ~0.4%, respectively (1, 2). Genetic-environmental interactions have been established between disease-associated major histocompatibility complex haplotypes and specific pathogen peptides that mimic disease self antigens (3, 4). The prevalence of MS and type 1 diabetes changes along north-south gradients, implicating ultraviolet light exposure and production of vitamin D3 in the skin (5–7), a hormone known to negatively regulate T cell function, the MS animal model experimental autoimmune encephalomyelitis (EAE), and spontaneous autoimmune diabetes in the non-obese diabetic (NOD) mouse (5, 8–11). However, molecular mechanisms for genetic-environmental interactions are poorly understood.

Salvage of glucosamine by the hexosamine pathway to UDP-GlcNAc is reported to suppress T cell function and EAE in mice by an unknown mechanism (12, 13). *De novo* biosynthesis of UDP-GlcNAc by the hexosamine pathway utilizes glucose, acetyl-CoA, glutamine, and UTP (see Fig. 1), key allosteric regulators of basic metabolism, suggesting regulation of UDP-GlcNAc supply is integrated with down-stream pathways requiring this sugar-nucleotide. In this regard, the Golgi pathway to β1,6GlcNAc-branched *N*-glycans is sensitive to cellular UDP-GlcNAc levels (14) and plays a role in T cell function (15). Mice deficient in Golgi UDP-GlcNAc:β1,6N-acetylglucosaminyltransferase V (Mgat5) display enhanced delayed-type hypersensitivity, spontaneous kidney autoimmunity after 1 year of age, and increased susceptibility to EAE (15).

Most transmembrane receptors on mammalian cells are modified by *N*-glycosylation en route to the cell surface. The *N*-acetylglucosaminyltransferases I, II, IV, and V, encoded by the genes Mgat1, -2, -4a/b, and -5, act sequentially to transfer *N*-acetyl-D-glucosamine (GlcNAc) from UDP-GlcNAc to *N*-glycan intermediates in the medial Golgi, producing mono, bi-, tri-, and tetra-antennary *N*-glycans, respectively (16, 17) (see Fig. 1). Branching and particularly the β1,6GlcNAc-branched tetra-antennary *N*-glycans are sub-saturating, as Mgat1, -2, -4, and -5 display decreasing affinities for UDP-Glc-

* This research was supported by grants from Canadian Institutes for Health Research (to J. W. D.) and from the National Multiple Sclerosis Society, the Juvenile Diabetes Research Foundation, the Wadsworth Foundation, and NIAID, National Institutes of Health (to M. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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[3] The abbreviations used are: MS, multiple sclerosis; MS/MS, tandem mass spectroscopy; NOD, non-obese diabetic; TCR, T cell receptor; EAE, experimental autoimmune encephalomyelitis; L-PHA, *P. vulgaris* leukoagglutinin; SW, swainsonine; TCR, T cell receptor; FACS, fluorescence-activated cell sorter; CFSE, carboxyfluorescein diacetate succinimidyl ester; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; ELISA, enzyme-linked immunosorbent assay; MBP, myelin basic protein; IFN, interferon; IL, interleukin; FITC, fluorescein isothiocyanate.

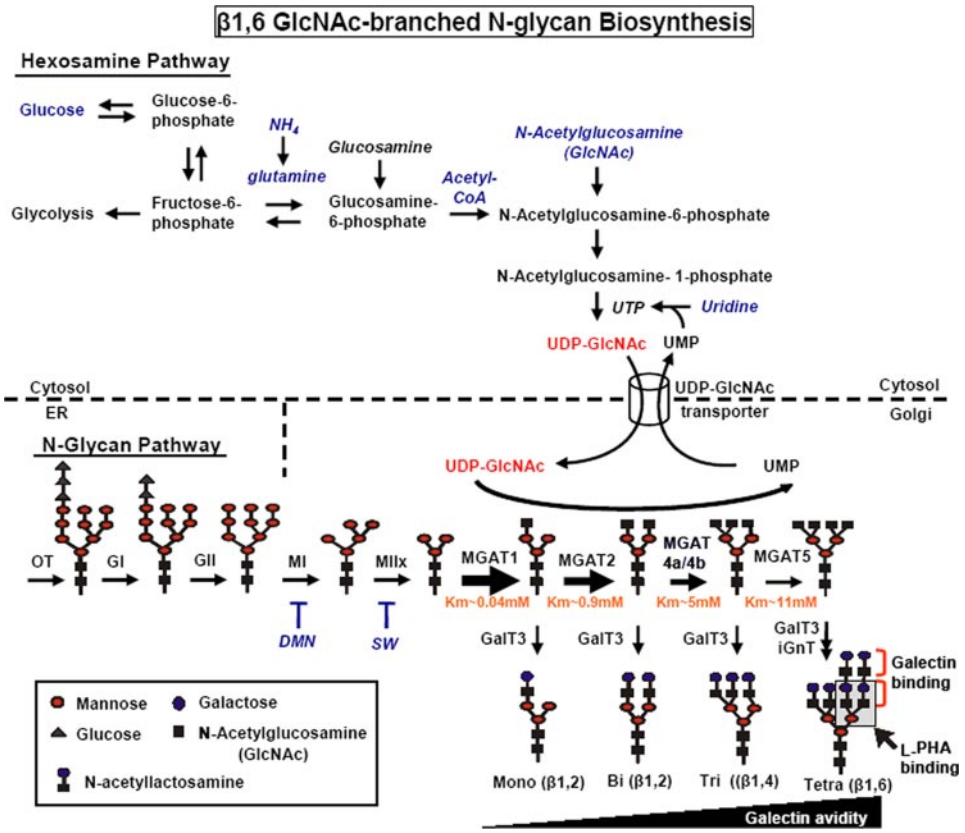

FIGURE 1. **Regulation of GlcNAc-branched *N*-glycan biosynthesis by the hexosamine and *N*-glycan pathways.** UDP-GlcNAc is required by the *N*-acetylglucosaminyltransferases Mgat1, -2, -3, -4, and -5 and iGnT. The size of the *arrows* for Mgat1, -2, -4, and -5 depicts the relative affinity for UDP-GlcNAc, with K_m *below* the *arrows*. Cytosolic UDP-GlcNAc enters the Golgi via antiporter exchange with Golgi UMP, a reaction product of the *N*-acetylglucosaminyltransferases. Galectins bind *N*-acetyllactosamine, with avidity increasing in proportion to the number of *N*-acetyllactosamine units (*i.e.* GlcNAc branching). β1,6GlcNAc-branching by Mgat5 promotes poly-*N*-acetyllactosamine production, further enhancing avidity for galectins. *ER*, endoplasmic reticulum; *DMN*, deoxymannojirimycin; *GI*, glucosidase I; *GII*, glucosidase II; *MI*, mannosidase I; *MIIx*, mannosidase IIx; *GalT3*, galactosyltransferase3. A bypass pathway involving Mgat1 activity before mannosidase II is not shown.

NAc, and enzyme concentrations also decline across the pathway (see Fig. 1) (17). Galectins, a family of *N*-acetyllactosamine binding animal lectins, bind cell surface glycoproteins and form a molecular lattice that negatively regulates lateral movement and endocytic loss of surface receptors and transporters (15, 18–21). Galectins bind to *N*-glycans on surface glycoproteins with affinities proportional to GlcNAc-branching (Fig. 1) (15, 18, 19). β1,6GlcNAc-branched *N*-glycans are preferentially extended by poly-N-acetyllactosamine (*i.e.* (Galβ1,4GlcNAcβ1,3-)$_n$), further increasing avidity for galectins. However, increasing the proportion of tri-antennary structures in Mgat5-deficient cells is sufficient to rescue defects in galectin binding and surface retention of receptors (22). In addition, the number of *N*-glycans, an encoded feature of protein sequence (N*X*(S/T)), contributes to galectin avidity and regulates surface residency of receptors in a selective manner (22). With increasing hexosamine flux of UDP-GlcNAc through the Golgi to *N*-glycan GlcNAc branching, surface galectin binding and retention of glycoproteins with high *N*-glycan multiplicity (epidermal growth factor, insulin-like growth factor, platelet-derived growth factor, and basic fibroblast growth factor receptors) are enhanced before those with lower multiplicity (transforming growth factor-β receptor, CTLA-4, and GLUT-4) (22). This provides a mechanism for metabolic regulation of cellular transitions between growth (*i.e.* epidermal growth factor, insulin-like growth factor, platelet-derived growth factor, and basic fibroblast growth factor receptors) and arrest signaling (*i.e.* transforming growth factor-β receptor, CTLA-4, and GLUT-4) (22).

β1,6GlcNAc-branched *N*-glycans attached to the T cell receptor enhance binding to galectin-3, limiting TCR clustering at the immune synapse and increasing agonist thresholds for TCR signaling (15, 22, 23). After TCR activation, endocytosis rates are increased and Src-family kinases and phosphatidylinositol 3-kinase/Erk stimulate hexosamine flux and Golgi processing to β1,6GlcNAc-branched *N*-glycans, thereby enhancing surface retention of the growth suppressor CTLA-4 (22). Therefore, β1,6GlcNAc-branched *N*-glycans regulate different receptors and at two distinct phases of the T cell response, both serving to suppress T cell activation and autoimmune mechanisms. Here we demonstrate that surface β1,6GlcNAc-branched *N*-glycans on T cells are regulated by the nutrient environment and metabolite supply to the hexosamine pathway. Hexosamine pathway supplements suppress TCR sensitivity, T_H1 differentiation, and autoimmunity by increasing *N*-glycan GlcNAc-branching in T cells. Our results indicate that genetic and environmental contributions to metabolic homeostasis and Golgi processing regulate *N*-glycan branching, T cell function, and autoimmunity and suggest new avenues for prevention and treatment of autoimmune disease (Fig. 7).

EXPERIMENTAL PROCEDURES

FACS Analysis and in Vitro Proliferation Assays—PL/J mice were congenic at backcross six from our original 129/Sv Mgat5$^{-/-}$ mice (24). NOD mice were obtained from Jackson laboratories at 5 weeks of age. 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-CTLA-4 (UC10-4B9), anti-CD28 (37.51), and anti-CD69 (H1.2F3) from eBioscience, *Phaseolus vulgaris* leukoagglutinating lectin (L-PHA, 4 μg/ml), *Lycopersicon esculentum* agglutinating lectin (20 μg/ml), and 7-aminoactinomycin D (1 μg/ml) from Sigma. Purified CD3$^+$ T cells (R&D Systems) were labeled with 5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) in phosphate-buffered saline for 8

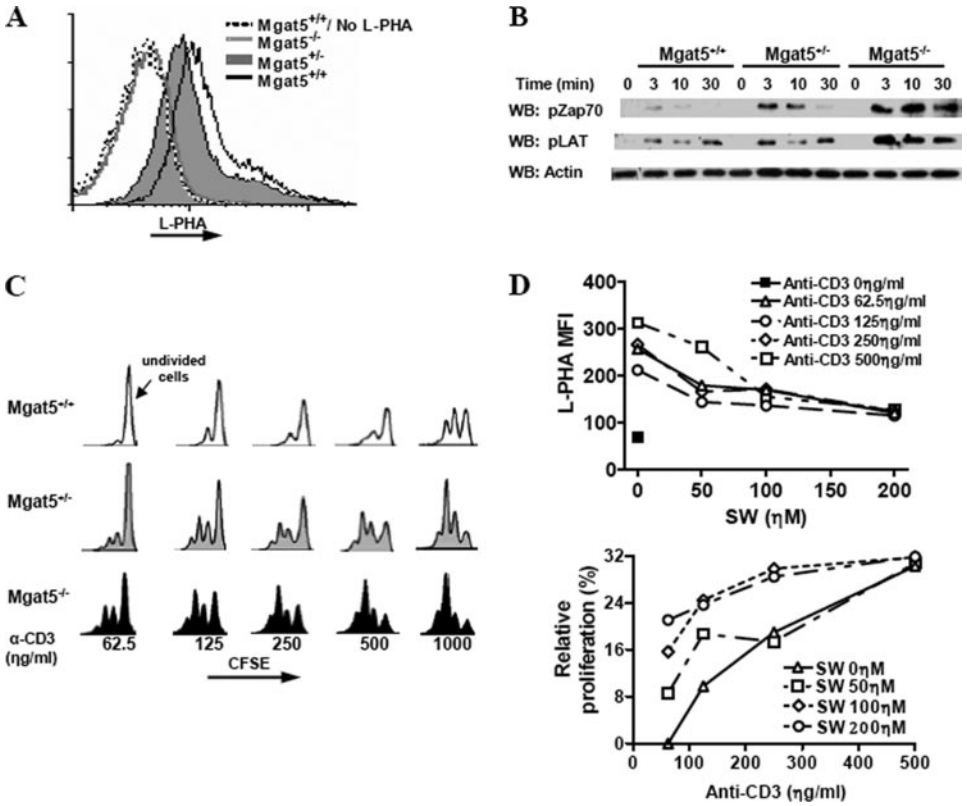

FIGURE 2. **β1,6GlcNAc-branched *N*-glycan expression and dose-dependent regulation of T cell function.** *A*, L-PHA FACS analysis of $CD4^+$ T cells from the indicated genotypes. Background fluorescence in L-PHA-stained $Mgat5^{-/-}$ $CD4^+$ T cells is the same as non-L-PHA-stained $Mgat5^{+/+}$ $CD4^+$ T cells, confirming previous observations that L-PHA specifically binds β1,6GlcNAc-branched *N*-glycans (15). *B*, purified $CD3^+$ T cells of the indicated genotypes were incubated at 37 °C with anti-CD3 antibody-coated microbeads for various times, lysed, and Western-blotted (*WB*). *C*, purified $CD3^+$ T cells were labeled with CFSE, stimulated for 3 days, and analyzed by FACS. The results shown are gated on $CD4^+$ T cells. *D*, purified $CD3^+$ T cells were labeled with CFSE (*lower panel*) or left unstained (*upper panel*), stimulated for 5 days in the presence or absence of SW, and analyzed by FACS. Shown is L-PHA mean fluorescence intensity (*MFI*) (*upper panel*) and percentage increase in the number of proliferating $CD4^+$ T cells relative to cells stimulated with 62.5 ng/ml anti-CD3 (*lower panel*). All data are gated on $CD4^+$ T cells. The results are representative of at least three independent experiments.

min at room temperature and stimulated with plate-bound anti-CD3ε (2C11, eBioscience) in the presence or absence of GlcNAc and/or swainsonine (SW) (Sigma). Jurkat T cells were cultured in either glutamine-free RPMI 1640, 10% fetal bovine serum, 10/20 mM glucose, or glucose/glutamine-free Dulbecco's modified Eagle's base medium supplemented with 10% fetal bovine serum, 1.5 mM glucose. The indicated monosaccharides and/or metabolites were added daily except glucose, which was added only at time 0 and were titrated until a plateau was reached in L-PHA staining or toxicity was observed. The plateau or highest non-toxic dose is shown.

TCR Signaling—1×10^6 purified splenic $CD3^+$ T cells from $Mgat5^{+/+}$, $Mgat5^{+/-}$, and $Mgat5^{-/-}$ mice or from purified T cell cultures incubated in the presence or absence of GlcNAc (80 mM) and/or swainsonine (0.25 μM) for 72 h were mixed with 5×10^6 polystyrene beads (6 μm, Polysciences) coated at 4 °C overnight with 0.5 μg/ml anti-CD3ε antibody (2C11, eBioscience) and pelleted at 5000 rpm for 15 s, incubated at 37 °C for the indicated times, and then solubilized with ice-cold 50 mM Tris, pH 7.2, 300 mM NaCl, 1.0% Triton X-100, protease inhibitor mixture (Roche Applied Science), and 2 mM orthovanadate for 20 min. Cell lysates were separated on Nupage 10% Bis-Tris gels (Invitrogen) under reducing conditions, transferred to polyvinylidene difluoride membranes, and immunoblotted with rabbit anti-phospho-Zap70 antibody (Ab; Cell Signaling Technology), rabbit anti-phospho-LAT Ab (Upstate), and/or anti-actin Ab (Santa Cruz).

MS/MS Mass Spectroscopy—For MS/MS mass spectroscopy to determine sugar nucleotide levels, Jurkat T cell pellets (20×10^6 cells) were resuspended in 300 ml of cold methanol:water (1:1) solution containing maltose as an internal standard, vortexed for 10 s, and then pipetted into tubes containing 600 ml of chloroform:methanol (3:2). Maltose (100 pmol) was added to each sample as an internal standard. Samples were vortexed for 1 min and then centrifuged at 14,000 rpm for 5 min at 4 °C. Supernatants were collected, and an equal volume of chloroform:methanol (1:1) was added followed by a second extraction. The pooled aqueous fraction containing the hydrophilic metabolite was dried with a SpeedVac, passed over C18 SepPak in water, dried, and stored at −80 °C. Before injection, the samples were dissolved in 100 ml of methanol:water (1:1). The samples were injected at 150 ml/h and analyzed on a 4000QTRAP (Mass Spectrometer (SCIEX). The metabolites were identified by their transitions in MS/MS and quantified using the Analyst Software (Applied Biosystems- SCIEX), which measured the area under the curve for major fragment ion (M2) corresponding to each parent ion (M1). Collision energy, declustering potential, and ion spray voltage were optimized for each metabolite. UDP-GlcNAc (M1 = 605.9 and M2 = 385.0 with setting declustering potential −40, collision energy −38, and ion spray voltage −3500) and UDP-Gal (M1 = 564.9 and M2 = 323.0 with setting declustering potential +80, collision energy +23, ion spray voltage +5500) were measured in negative and positive, respectively. Maltose was measured in negative mode (M1 = 341 and M2 = 161 with setting declustering potential −40, collision energy −13, and ion spray voltage −4000). Standards for maltose, UDP-GlcNAc, and UDP-Gal showed sensitivity to 15 pmol and linearity to 10 nmol. Data are reported as UDP-HexNAc and UDP-Hex due to potential interconversion of UDP-GlcNAc to UDP-GalNAc and UDP-Gal to UDP-Glc by 4′-epimerase activity, sugar nucleotides not distinguished in M1.

In Vivo GlcNAc Treatment—GlcNAc was administered orally in age- and sex-matched PL/J littermate mice by adding GlcNAc to their drinking water at various concentrations. Fresh GlcNAc was given daily for 5 days, with the amount consumed approximated by determining the volume remaining

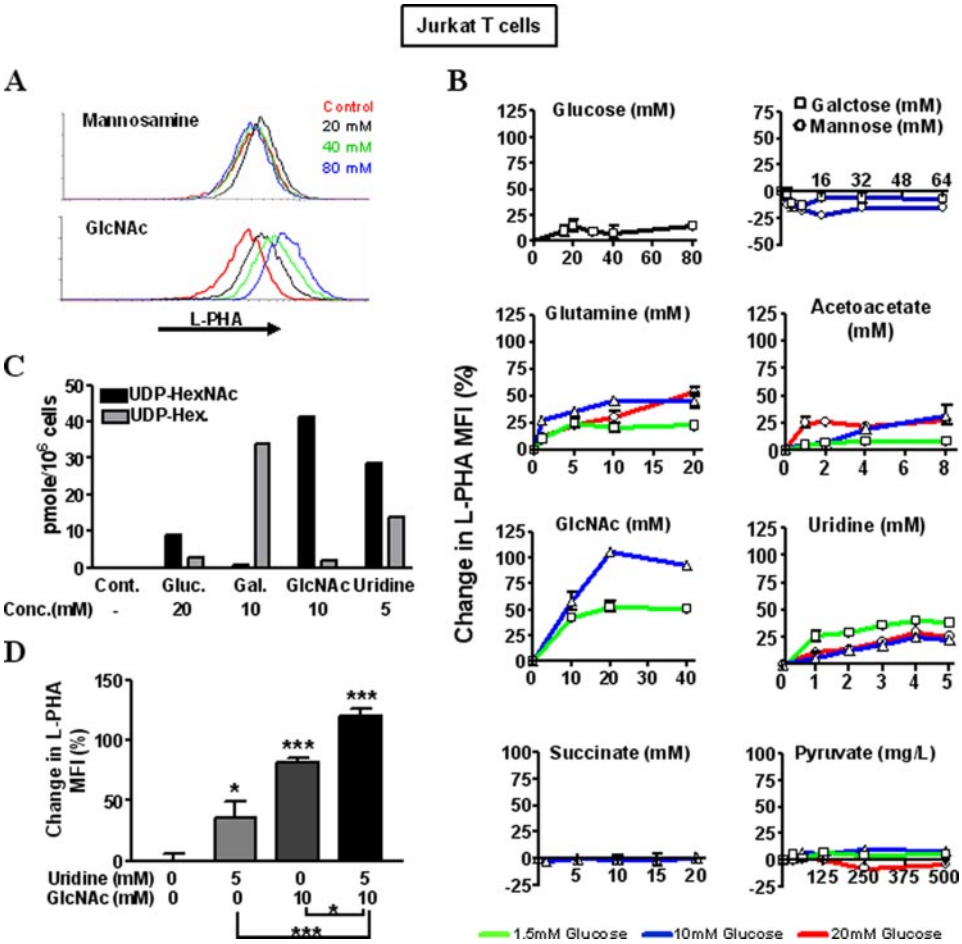

FIGURE 3. **Metabolite supply to the hexosamine pathway regulates β1,6GlcNAc-branched *N*-glycan expression in Jurkat T cells.** *A–D*, the indicated monosaccharides and metabolites were cultured with Jurkat T cells for 3 days, stained with L-PHA-FITC in triplicate, and analyzed by FACS (*A*, *B*, and *D*) or lysed and analyzed by MS/MS mass spectroscopy for sugar-nucleotide expression (*C*). *Green*, *blue*, and *red lines* in *B* refer to altered glucose concentrations in the culture media as indicated; all others were cultured in 10 mM glucose. UDP-HexNAc and UDP-Hex levels in non-supplemented cells were below the detection level of our assay. Detection via high performance liquid chromatography indicates GlcNAc supplementation increases the UDP-HexNAc: UDP-Hex ratio up to ~6-fold in TCR-activated mouse T cells (22). *Error bars* are present in (*A*, *B*, and *D*) and represent the means ± S.E. of triplicate staining. *p* values in *D* are by analysis of variance and the Newman-Keuls multiple comparison test with non-supplemented cells or as indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

after 24 h. After 5 days of treatment, harvested splenocytes were stained and analyzed by FACS.

Cytokine ELISA—Supernatant from splenocyte and/or T cell cultures stimulated with myelin basic protein (MBP) and/or anti-CD3ϵ (2C11, eBioscience) in the presence or absence of GlcNAc and/or SW were tested for IFN-γ, IL-6, and/or IL-4 levels by ELISA. Microtiter plates were coated with 50 μl of anti-IFN-γ (1 μg/ml, clone AN-18; eBioscience), anti-IL-6 (1.5 μg/ml, clone MP5–20F3; eBioscience), or anti-IL-4 (2 μg/ml, clone 11B11; eBioscience) overnight at 4 °C. Supernatants were applied at 50 μl/well and incubated for 2 h at room temperature. Captured cytokines were detected using biotinylated anti-IFN-γ (1 μg/ml, clone R4–6A2; eBioscience), anti-IL-6 (1 μg/ml, clone MP5–32C11; eBioscience), or anti-IL-4 (1 μg/ml, clone BVD6–24G2; eBioscience) and detected using avidin horseradish peroxidase (eBioscience) at 1:500× dilution and *o*-phenylenediamine dihydrochloride tablets (Sigma) according to the manufacturer's protocols. Recombinant IFN-γ, IL-6, or IL-4 (eBioscience) was used as a standard.

Adoptive Transfer EAE and NOD Diabetes—Adoptive transfer EAE was induced by subcutaneous immunization of wild-type PL/J mice with 100 μg of bovine MBP (Sigma) emulsified in Complete Freund's Adjuvant containing 4 mg/ml heat-inactivated *Mycobacterium tuberculosis* (H37 RA; Difco) distributed over three spots on the hind flank. Splenocytes were harvested after 11 days and stimulated *in vitro* with 50 μg/ml MBP in the presence or absence of 40 mM GlcNAc (Sigma) added daily. After 96 h of incubation, $CD3^+$ T cells were purified by negative selection (R&D Systems) and injected intraperitoneally into naïve PL/J $Mgat5^{+/-}$ recipient mice. Trypan blue exclusion determined <5% dead cells under both culture conditions. Mice were scored daily for clinical signs of EAE over the next 30 days with the observer blinded to treatment conditions. Mice were examined daily for clinical signs of EAE and scored in a blinded fashion as follows: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness or paralysis and hindlimb paralysis; 5, moribund or dead. GlcNAc was administered orally to female NOD mice by adding fresh GlcNAc at a concentration of 250 μg/ml to the drinking water every 3 days. Spontaneous diabetes in wild-type NOD mice was diagnosed after two consecutive positive tests for glycosuria of ≥2000 mg/dl by Chemstrip (Accu-chek, Roche Applied Science) 1 week apart.

RESULTS

T Cell Thresholds and Fractional Changes in β1,6GlcNAc Branching—Conditional or metabolic regulation of T cell activation thresholds could be expected to be sensitive to small incremental changes in *N*-glycan branching. To test this hypothesis we compared the agonist sensitivity of primary T cells from $Mgat5^{+/+}$, $Mgat5^{+/-}$, and $Mgat5^{-/-}$ mice. $Mgat5^{+/-}$ T cells show an ~20–25% reduction in β1,6GlcNAc-branched *N*-glycans relative to $Mgat5^{+/+}$ cells as indicated by L-PHA binding, a plant lectin specific for these structures (Fig. 2*A*) (15, 23). Stimulation of purified $CD3^+$ T cells with anti-CD3- coated microbeads demonstrates that $Mgat5^{+/-}$ cells are intermediate compared with $Mgat5^{+/+}$ and $Mgat5^{-/-}$ cells for TCR signaling as shown by enhanced phosphorylation of Zap-70 and LAT (Fig. 2*B*). $Mgat5^{+/-}$ T cells are similarly intermediate for TCR-mediated proliferation, as

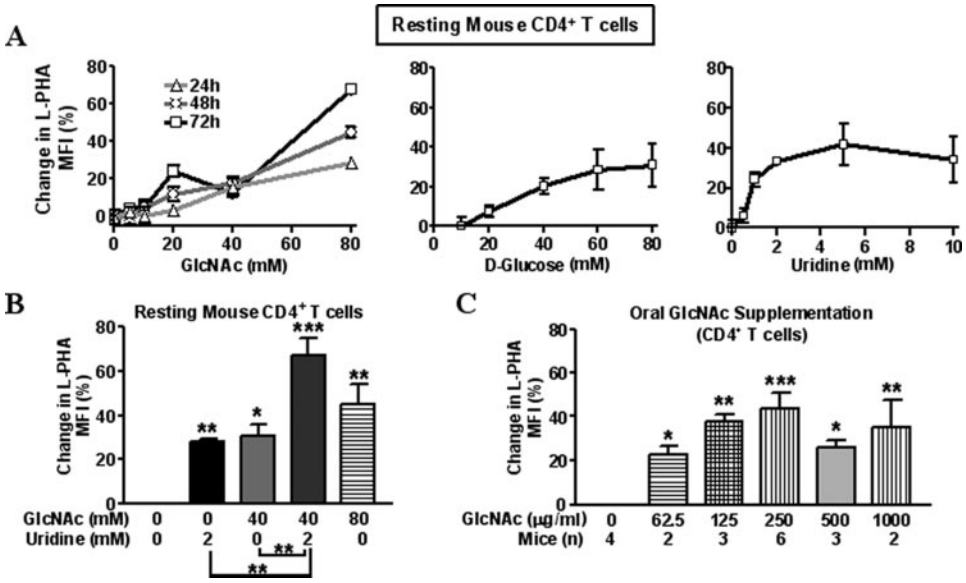

FIGURE 4. **Metabolite supply to the hexosamine pathway regulates β1,6GlcNAc-branched *N*-glycan expression in resting primary mouse T cells.** *A* and *B*, the indicated monosaccharides and metabolites were cultured with unstimulated mouse splenocytes for 3 days, stained with L-PHA-FITC in triplicate, and analyzed by FACS. The results are representative of at least three independent experiments. *Error bars* are present in all panels and represent the means ± S.E. of triplicate samples. *C*, splenocytes from Mgat5$^{+/-}$ mice orally supplemented with GlcNAc for 5 days via their drinking water at the indicated concentrations were stained with L-PHA-FITC in triplicate and analyzed by FACS. On average, mice drank ~4.6 ± 0.25 ml/day, estimated by determining the amount of GlcNAc solution remaining daily. For a 28.5-g mouse, the approximate mg/kg doses received is 10, 20, 40, 80, and 160. Data shown are an average from two independent experiments, each with triplicate staining. *n* = number of mice. *Error bars* are the means ± S.E. *p* values in *B* and *C* are by analysis of variance and the Newman-Keuls multiple comparison test with non-supplemented cells/mice or as indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

shown by tracking cell division with CFSE (Fig. 2*C*). To confirm that small reductions in β1,6GlcNAc-branched *N*-glycans enhance TCR sensitivity, we treated wild type cells with minimal concentrations of SW. SW is a specific inhibitor of mannosidase II that blocks GlcNAc branching in *N*-glycans and is known to enhance T cell proliferation and T_H1 differentiation (23, 25). At low levels of anti-CD3 stimulation, partial reductions in β1,6GlcNAc-branched *N*-glycans by low doses of swainsonine significantly enhance wild type T cell proliferation (Fig. 2*D*) (25, 26). Thus, changes in T cell activation thresholds can be readily detected with as little as ~20–25% variations in β1,6GlcNAc-branched *N*-glycans.

Metabolic Flux through the Hexosamine Pathway Regulates β1,6GlcNAc-branched N-Glycans in T Cells—The expression of tri (*i.e.* β1,4)- and tetra (*i.e.* β1,6)-antennary GlcNAc-branched *N*-glycans in cultured cells is sensitive to changes in the intracellular concentration of UDP-GlcNAc (14, 22), which enters the Golgi via UDP-GlcNAc transporters (27). The addition of GlcNAc to cultured cells supplements UDP-GlcNAc pools after uptake by bulk endocytosis, 6-phosphorylation, and conversion to UDP-GlcNAc (Fig. 1) (14, 22). Glucose, glutamine, acetyl-CoA, and UTP are metabolites required by the hexosamine pathway for *de novo* UDP-GlcNAc biosynthesis (Fig. 1). TCR activation of mouse T cells increases Mgat5 gene expression (15), glucose uptake (28), UDP-HexNAc levels (22), and β1,6GlcNAc-branched *N*-glycans (23). GlcNAc supplementation of activated T cells further enhances UDP-HexNAc levels, increasing the UDP-HexNAc:UDP-Hex ratio up to ~6-fold (22). To determine whether hexosamine metabolite supplementation enhances expression of cell surface galectin ligands (*i.e. N*-glycan GlcNAc branching and poly-*N*-acetyllactosamine) in resting T cells, cells were titrated with various metabolites until a plateau was reached in L-PHA staining or toxicity was observed. Surface levels of β1,6GlcNAc-branched *N*-glycans (*i.e.* L-PHA staining) and poly-*N*-acetyllactosamine (indicated by staining with the plant lectin *L. esculentum* agglutinin) on non-TCR activated human Jurkat T cells were increased by supplementing the hexosamine pathway with high glucose, GlcNAc, acetoacetate, glutamine, ammonia, or uridine but not with control metabolites mannosamine, galactose, mannose, succinate, or pyruvate (Fig. 3, *A* and *B*; supplemental Fig. 1, *A* and *B*). Supplementing resting mouse *ex vivo* splenocytes with GlcNAc, glucose, or uridine *in vitro* significantly raised β1,6GlcNAc-branched *N*-glycans in CD4$^+$ T cells (Fig. 4*A*). In non-T cells, GlcNAc supplementation also increases tri-antennary structures (22), and these are likely also enhanced in supplemented T cells. MS/MS mass spectroscopy confirmed that GlcNAc, glucose, and uridine supplements raised intracellular UDP-HexNAc levels in non-TCR-activated Jurkat T cells (Fig. 3*C*). On a per cell basis, UDP-HexNAc levels in Jurkat T cells appear lower than that reported for Chinese hamster ovary cells (29); however, T cells are smaller and have a markedly higher nuclear to cytoplasmic ratio, making direct comparisons difficult. Co-supplementation of GlcNAc and uridine in resting Jurkat and wild type mouse T cells were additive and more effective than doubling the GlcNAc concentration (Figs. 3*D* and 4*B*). Supplementing the drinking water of Mgat5$^{+/-}$ or Mgat5$^{+/+}$ mice with GlcNAc increased β1,6GlcNAc-branched *N*-glycans up to ~40% on CD4$^+$ T cells *in vivo* (Fig. 4*C*; supplemental Fig. 2*A*). Taken together, these data indicate that production of high affinity galectin ligands (*e.g.* β1,6GlcNAc-branched *N*-glycans and poly-N-acetyllactosamine) are limited in T cells by the availability of key intermediates of glucose, lipid, nitrogen, and nucleotide metabolism.

Suppression of T Cell Function and Autoimmunity by the Hexosamine Pathway Is Dependent on N-Glycan GlcNAc Branching—Genetically induced reductions in T cell β1,6GlcNAc branching weaken the galectin lattice, enhancing agonist-induced TCR clustering and signaling, T_H1 differentiation (*i.e.* increased IFNγ and reduced IL-4 production), CTLA-4 endocytosis, T cell proliferation, and susceptibility to autoimmunity (Fig. 2, *A–C* and Refs. 15, 22, and 23). Consequently, metabolic enhancement of *N*-glycan GlcNAc branching, particularly β1,6GlcNAc-branching, should strengthen the galectin lattice

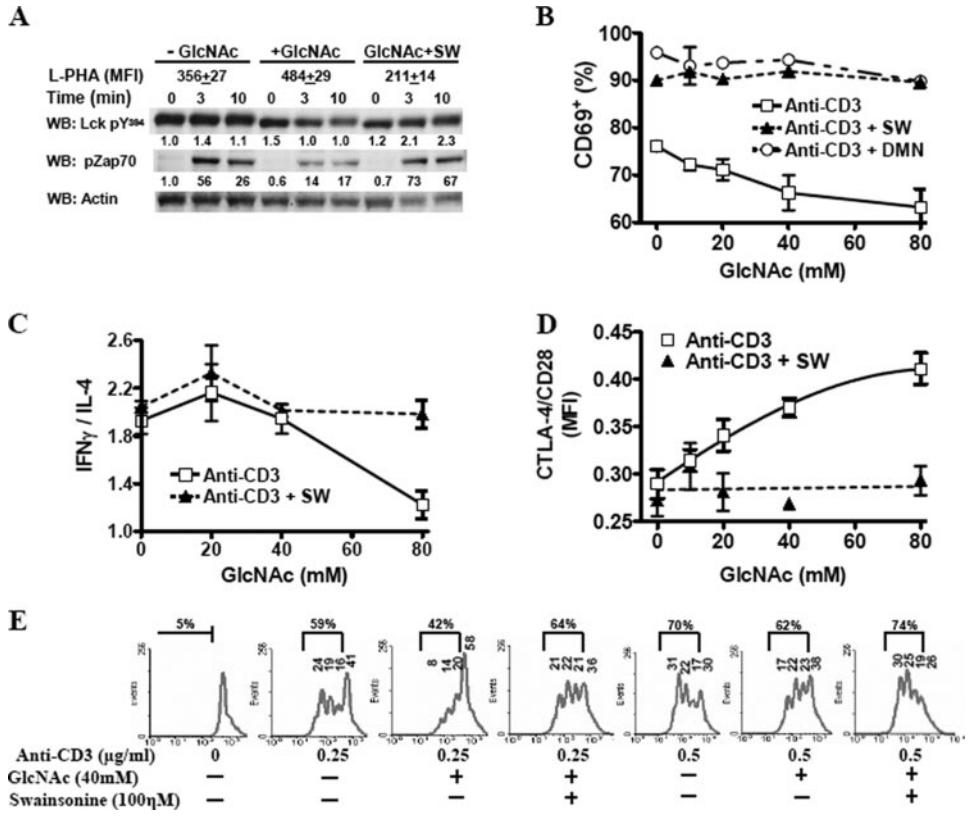

FIGURE 5. **Regulation of T cell function by the hexosamine pathway.** *A*, resting purified $CD3^+$ T cells from wild-type mice preincubated for 2 days in the absence or presence of 80 mM GlcNAc and/or 250 nM SW as indicated were stained with L-PHA-FITC in triplicate or incubated at 37 °C with anti-CD3 antibody-coated microbeads for various times, lysed, and Western-blotted (*WB*). Bands quantified using Gel Pro Analyzer software were first normalized to the resting vehicle-treated lane and then to actin. *Numbers* are shown *below* each *band*. Note that phosphorylation of Lck Tyr^{394} is reduced at 3 and 10 min relative to rest in GlcNAc-treated but not vehicle and GlcNAc+SW- treated cells. *B–D*, wild-type $CD3^+$ T cells were stimulated with anti-CD3 antibody in the presence or absence of GlcNAc, SW, and/or deoxymannojirimycin (*DMN*) as indicated for 2 days (*C*), 3 days (*B*), or 4 days (*D*). *B*, cells were analyzed by FACS for CD69 staining. The results are representative of three independent experiments. *Error bars* are present in all panels and represent the means ± S.E. of triplicate samples. Anti-CD3 = 62.5 ng/ml, SW = 250 nM, deoxymannojirimycin = 2 mM. *C*, harvested supernatant from triplicate wells were analyzed by ELISA for IFNγ and IL-4 and shown as a ratio of IFNγ to IL-4, eliminating the effects of GlcNAc and/or swainsonine on T cell number. Similar data for IFNγ was obtained at 3 days using splenocytes (see supplemental Fig. 3*C*). Anti-CD3 = 62.5 ng/ml, SW = 250 nM. *Error bars* indicate the means ± S.E. of triplicate samples. *D*, cells were analyzed by FACS for CTLA-4 and CD28 staining, and data are shown as a ratio of CTLA-4/CD28 mean fluorescence intensity (*MFI*). The results are representative of three independent experiments. *Error bars* indicate the means ± S.E. of triplicate samples. *E*, cells were analyzed by FACS for CFSE staining as indicated after 3 days in culture. *Numbers above* the *peaks* indicate the percentage of the total. Data shown are gated on $CD4^+$ T cells. The results are representative of five independent experiments.

and negatively regulate these phenotypes. Hexosamine supplementation of wild-type resting T cells with GlcNAc inhibited anti-CD3-induced phosphorylation of Lck at activating Tyr^{394} and Zap-70 (Fig. 5*A*). TCR signal strength regulates expression of the T cell activation marker CD69 as well as production of IFNγ relative to IL-4 (30), and as expected, both are inhibited by hexosamine supplementation (Fig. 5, *B* and *C*; supplemental Fig. 3, *A–C*). CTLA-4 retention at the surface of blasting T cells is promoted by TCR signaling-mediated increases in hexosamine flux and Golgi processing to β1,6GlcNAc-branched *N*-glycans (22). GlcNAc supplementation significantly increases surface levels of CTLA-4 relative to CD28 in T cell blasts (Fig. 5*D*), receptors that compete for binding to CD80/86 on antigen-presenting cells to positively and negatively regulate growth arrest, respectively (31). Tracking cell division of purified T cells using CFSE labeling demonstrates that hexosamine supplementation inhibits T cell proliferation without inducing cellular toxicity as measured by 7-aminoactinomycin D (Fig. 5*E*; supplemental Fig. 3, *D–F*). Very importantly, all of the observed effects of hexosamine supplementation on T cell function are dependent on *N*-glycans, as they could be reversed by blocking *N*-glycan GlcNAc branching beyond mono-antennary with SW and/or the mannosidase I inhibitor deoxymannojirimycin (*DMN*, Fig. 1) (Figs. 5, *A–E*; supplemental Figs. 2*B* and 3, *B*, *C*, *E*, and *F*). These data demonstrate that hexosamine supplementation controls three temporally distinct autoimmune regulatory phenotypes, namely T cell activation thresholds, T_H1 differentiation, and CTLA-4 endocytosis, by enhancing GlcNAc branching in *N*-glycans.

Next, we determined whether hexosamine-induced changes in GlcNAc-branched *N*-glycans suppresses T cell-mediated autoimmunity. The MS model EAE may be induced by adoptive transfer of myelin antigen-specific T cells into naïve mice, leading to inflammatory demyelination of axons and progressive motor weakness. Splenocytes from MBP-immunized wild-type PL/J mice were re-stimulated *in vitro* with MBP in the presence or absence of GlcNAc, then transferred to naïve mice for induction of EAE. Re-stimulation in the presence of GlcNAc increased β1,6GlcNAc-branched *N*-glycans and poly-*N*-acetyllactosamine expression and inhibited production of the T_H1 cytokine IFN-γ while promoting secretion of the T_H2 inducing/T_H1 inhibitory cytokine IL-6 (Fig. 6, *A* and *B*) (32). After adoptive transfer of the T cells into naïve PL/J mice, mice receiving the GlcNAc-supplemented cells showed dramatically reduced incidence and severity of EAE compared with mice given untreated cells (Fig. 6*C*).

To determine whether oral GlcNAc supplementation inhibits spontaneous autoimmunity, we supplemented the drinking water of female NOD mice at 250 μg/ml, a concentration that maximally raises β1,6GlcNAc-branched *N*-glycans in $CD4^+$ T cells *in vivo* (Fig. 4*C*). GlcNAc supplementation starting between the 5th and 6th week of age significantly reduced development of autoimmune diabetes (Fig. 6*D*). Insulitis begins at 3–4 weeks of age in NOD mice (11), suggesting that initiation of GlcNAc supplementation before insulitis may further reduce development of diabetes.

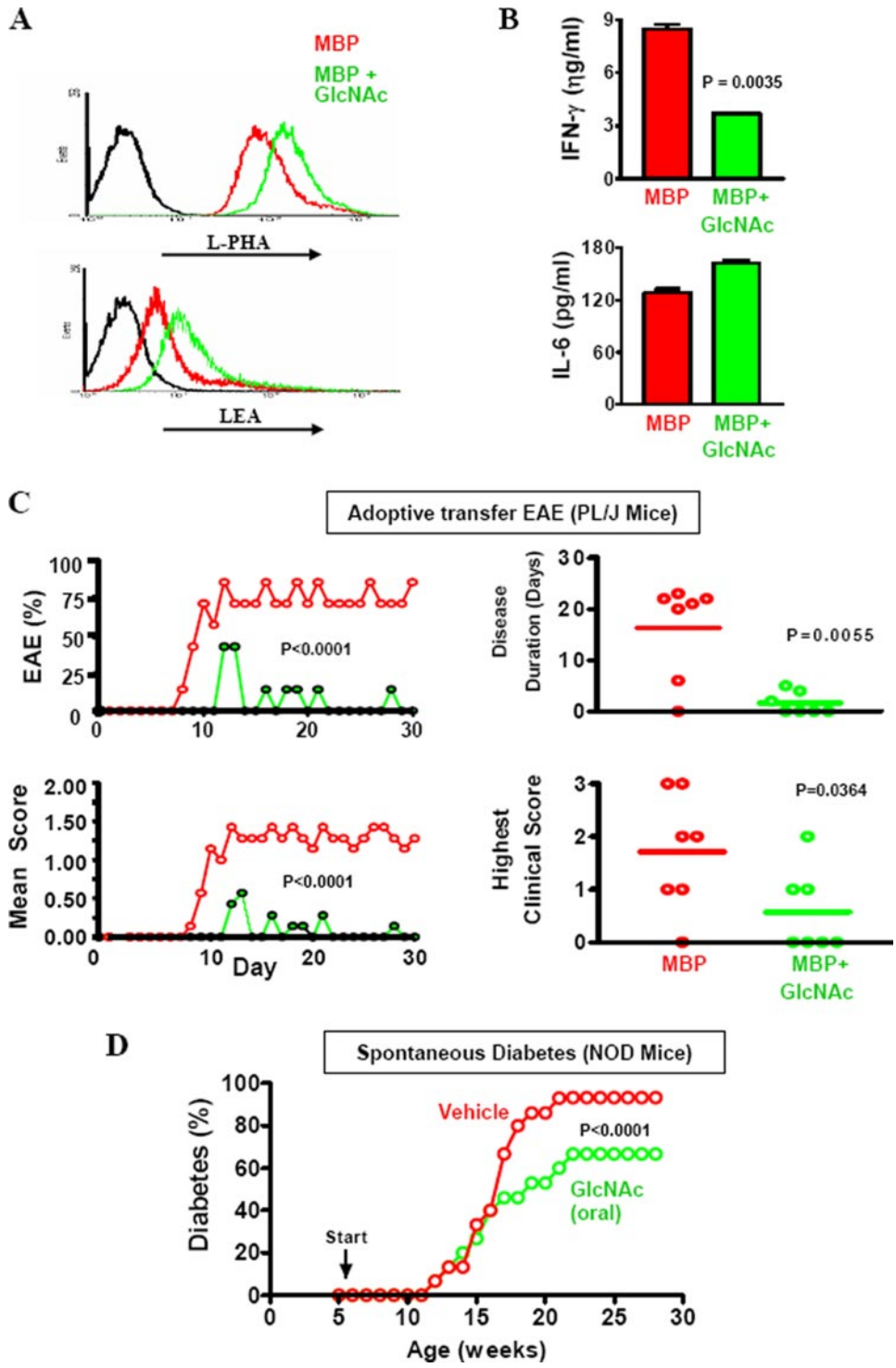

FIGURE 6. **Regulation of autoimmunity by the hexosamine pathway.** *A–C*, splenocytes were isolated from wild-type mice 11 days after immunization with MBP+ complete Freund's adjuvant (CFA) and re-stimulated *in vitro* with MBP for 4 days in the presence (*green*) or absence (*red*) of GlcNAc (40 mM). *A*, cells were stained with L-PHA-FITC and *L. esculentum* agglutinin (*LEA*)-FITC (a lectin specific for poly-*N*-acetyllactosamine) and analyzed by FACS. The *black line* represents autofluorescence. *B*, harvested supernatants were analyzed by ELISA for IFNγ and IL-6 production. *Error bars* indicate the means ± S.E. of duplicate samples, and *p* values were determined by Student's *t* test. IL-4 was below ~30 pg/ml, the detection limit of our ELISA. *C*, 3.6 million $CD3^+$ T cells were injected into naïve $Mgat5^{+/-}$ mice ($n = 7$ for each condition) and scored for signs of EAE daily for 30 days. *D*, the drinking water of female NOD mice was supplemented with ($n = 15$) or without ($n = 15$) 250 μg/ml GlcNAc starting between 5 and 6 weeks of age. Mice were assessed for glycosuria weekly, with diagnosis of diabetes made after two consecutive positive tests of ≥2000 mg/dl glucose by Chemstrip 1 week apart. *p* values for EAE and diabetes incidence was determined by Fisher's exact test. *p* values for EAE mean score, disease duration, and highest clinical score were determined by the Mann-Whitney test.

Others have demonstrated that *in vitro* and/or *in vivo* supplementation of mice with glucosamine inhibits T cell activation and T_H1 responses as well as graft rejection and EAE, but the mechanism is unknown (12, 13). GlcN enters the hexosamine pathway after 6-phosphorylation (Fig. 1). Our data demonstrate that GlcN increases β1,6GlcNAc branching in T cells, with maximal changes occurring at ~100–150 μM (supplemental Fig. 1*C*), the same concentration that others have found inhibit proliferation and T_H1 differentiation (13). Taken together, these data indicate key metabolites shared by hexosamine, glycolysis, lipid (free fatty acids to acetyl-CoA), amino acid (*i.e.* ammonia/glutamine) and nucleotide metabolism conditionally regulate thresholds for T cell function and autoimmunity by altering GlcNAc branching in *N*-glycans.

DISCUSSION

GlcNAc branching in *N*-glycans titrates binding to galectins, forming a molecular lattice at the cell surface that negatively regulates TCR sensitivity, T_H1 differentiation, CTLA-4 endocytosis, and the threshold to autoimmunity (15, 22, 23). Here we demonstrate that T cell function and autoimmunity are regulated by the nutrient environment and metabolite supply to the hexosamine pathway in an *N*-glycan GlcNAc-branching-dependent manner. This implies that genetic contributions to intracellular metabolic homeostasis coupled with the nutrient environment regulate GlcNAc-branching in *N*-glycans and, thereby, autoimmune susceptibility. Autoimmunity is a complex trait disease where genetic susceptibility is modulated by the environment via unclear mechanisms. In this regard metabolic regulation of GlcNAc branching provides an environmental mechanism to influence genetic predisposition to autoimmunity (Fig. 7).

GlcNAc and GlcN supplement the hexosamine pathway (Fig. 1),

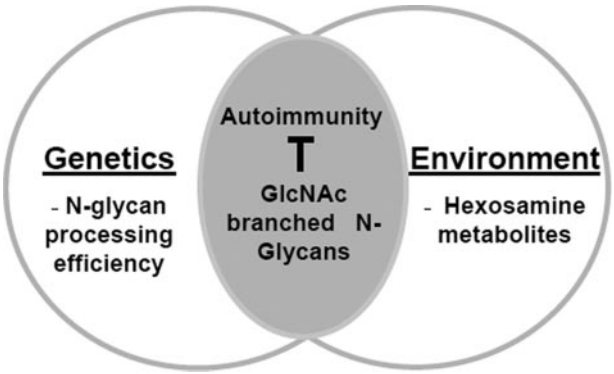

FIGURE 7. **A model for environmental and genetic regulation of autoimmunity via GlcNAc-branched *N*-glycans.**

increase β1,6GlcNAc-branched *N*-glycans, and inhibit T cell function and autoimmunity (Figs. 3*B*, 4*A*, 5, *A–E*, and 6, *A–C*; supplemental Fig. 1*C*) (12, 13). GlcN enters cells via glucose transporters, allowing an ~200–400-fold reduction in effective concentration relative to GlcNAc, which is taken up by bulk endocytosis. However, maximal increases in β1,6GlcNAc-branched *N*-glycans are ~2–4-fold greater with GlcNAc than GlcN. Moreover, increasing concentrations of GlcN (>200 μM), unlike GlcNAc, reduces β1,6GlcNAc-branched *N*-glycans. GlcN-6-phosphate but not GlcNAc-6-phosphate is a potent inhibitor of glutamine-fructose-6-phosphoaminotransferase, the rate-limiting enzyme shunting fructose 6-phosphate into the hexosamine pathway (33). GlcN-6-phosphate may also exit the hexosamine pathway to enter glycolysis via deamination by glucosamine-6-phosphate deaminase (Fig. 1). These data suggest GlcNAc functions as a better inducer of β1,6GlcNAc branching because glutamine-fructose-6-phosphoaminotransferase product inhibition and/or enhanced GNPD activity may limit the effectiveness of GlcN. In this regard, GlcNAc is predicted to be a superior therapeutic agent for prevention and/or treatment of human autoimmunity. Indeed, 8 of 12 children with severe treatment-resistant inflammatory bowel disease had significant clinical improvement after ~2 years of treatment with 3–6 g/day of oral GlcNAc (34). Assuming a weight of 40–50 kg for these children, which was not reported in the study, the dose used was ~60–150 mg/kg/day, similar to the 20–160 mg/kg/day dose that maximally raises T cell β1,6GlcNAc branching and inhibits autoimmune diabetes in mice. No significant adverse effects were noted over ~2 years of treatment, suggesting GlcNAc therapy is likely to be a safe therapeutic approach in humans.

In addition to the *N*-glycan GlcNAc transferases, UDP-GlcNAc is a sugar-nucleotide donor for the Golgi *O*-glycan pathway (35) and cytoplasmic *O*-GlcNAc transferase (36). UDP-GlcNAc may also be converted to UDP-GalNAc, the sugar-nucleotide utilized by GalNAc transferases to initiate *O*-glycosylation at Ser/Thr residues. Our data do not exclude metabolic-induced changes in these or other pathways requiring UDP-GlcNAc or UDP-GalNAc. However, the *N*-glycan pathway-specific inhibitor SW reverses all observed effects of GlcNAc supplementation on T cell function (Figs. 2*D* and 5, *A–E*; supplemental Fig. 3, *B*, *C*, *E*, and *F*). Similarly, GlcNAc-dependent inhibition of receptor endocytosis is reversed by mutation of *N*-glycosylation sites (*i.e.* N*X*(S/T)) and by knock-out of Mgat1 (22). Thus, metabolic regulation of GlcNAc-branched *N*-glycans is necessary and sufficient to produce the observed autoimmune inhibitory T cell phenotypes (*i.e.* T cell activation thresholds, T_H1 differentiation, and CTLA-4 surface expression), identifying *N*-glycans as a major target of hexosamine mediated inhibition of autoimmunity.

β1,6GlcNAc-branched *N*-glycans are present on β1 integrins and negatively regulate cell adhesion and promote tumor cell motility (19, 24, 37). Substratum and cell-cell adhesion regulates several aspects of T cell activation and migration. Antibodies that disrupt binding of T cell-associated $\alpha_4\beta_1$ integrin to vascular cell adhesion molecule-1 on activated endothelium negatively regulates EAE and MS by blocking T cell recruitment to the central nervous system (38, 39). β1,6GlcNAc branching in β1 integrins negatively regulates cell adhesion (24, 37, 40) including inhibition of T cell binding to vascular cell adhesion molecule-1 *in vitro*,[4] which may limit T cell recruitment to the central nervous system *in vivo*. Mgat5-deficient macrophages have decreased motility and phagocytosis (19), a phenotype that may promote autoimmunity by inhibiting clearance of apoptotic cells (41).

Mutations in genes of apparently distinct pathways for TCR signal strength (42–44), T_H1 differentiation (45), and CTLA-4 expression (46) promote T cell-mediated autoimmunity. However, targets of *N*-glycan GlcNAc branching include TCR, CTLA-4, and other surface glycoproteins (15, 22, 23), and taken with our results, *N*-glycan processing appears to serve as an intermediate in the conditional regulation of the cellular immune system. The hexosamine/Golgi processing pathways regulate at least three temporally distinct T cell phenotypes (activation, T_H differentiation, arrest) (15, 22, 23). This suggests that the hexosamine and Golgi pathways have co-evolved with the larger regulatory network that controls the cellular immune system. With a better understanding of the hexosamine pathway and its regulation of the immune system *in vivo*, metabolic therapy of human autoimmune disease may prove to be a useful intervention (47).

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