Metabolic control of T cell immune response through glycans in inflammatory bowel disease

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Mucosal T lymphocytes from patients with ulcerative colitis (UC) were previously shown to display a deficiency in branched N-glycosylation associated with disease severity. However, whether this glycosylation pathway shapes the course of the T cell response constituting a targeted-specific mechanism in UC remains largely unknown. In this study, we demonstrated that metabolic supplementation of ex vivo mucosal T cells from patients with active UC with N-acetylglucosamine (GlcNAc) resulted in enhancement of branched N-glycosylation in the T cell receptor (TCR), leading to suppression of T cell growth, inhibition of the T helper 1 (Th1)/Th17 immune response, and controlled T cell activity. We further demonstrated that mouse models displaying a deficiency in the branched N-glycosylation pathway (Mgat5−/−, Mgat5+/−) exhibited increased susceptibility to severe forms of colitis and early-onset disease. Importantly, the treatment of these mice with GlcNAc reduced disease severity and suppressed disease progression due to a controlled T cell-mediated immune response at the intestinal mucosa. In conclusion, our human ex vivo and predilutional results demonstrate the targeted-specific immunomodulatory properties of this simple glycan, proposing a therapeutic approach for patients with UC.

T lymphocytes | T cell receptor | adaptive immune response | branched N-glycosylation | intestinal inflammation

Inflammatory bowel diseases (IBDs), encompassing Crohn’s disease and ulcerative colitis (UC), are chronic, relapsing, and life-long inflammatory disorders of the gastrointestinal tract affecting mainly young populations. The incidence of IBD is increasing worldwide, and the disease remains incurable, placing a heavy burden on populations by reducing patients’ quality of life and increasing disability (1). The current therapeutic strategies for IBD are limited by reduced effectiveness, high costs, and/or side effects. This scenario highlights the urgent need in the clinic of identifying novel molecular markers capable of being selectively targeted with new and optimized therapies. Future progress in IBD monitoring and therapy mostly depends on the identification of key mechanisms mediating intestinal inflammation that could be therapeutically targeted.

The immune system is tightly regulated by glycosylation, through the addition of carbohydrate structures (glycans) to key molecules (proteins) involved in innate and adaptive immune responses (2). The N-acetylglucosaminyltransferase V (GnT-V) is a glycosyltransferase encoded by the human MGAT5 gene that catalyzes the synthesis of β1,6-N-acetylglucosaminyl GalNAc) branched N-glycans, which are known to play pivotal roles in many glycoproteins in cancer (3–6) and also in T cell activity and function (7, 8). In homeostasis and self-tolerance, T cell activation [via T cell receptor (TCR) signaling] induces up-regulation of the MGAT5 gene, which, in turn, leads to GnT-V-mediated glycosylation of the TCR (9). Consequently, it can promote growth arrest of T cells early, by raising T cell activation thresholds via limiting TCR clustering at the immune synapse (and restricting TCR signaling), and, later, by increasing surface retention of growth inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) (9).

In fact, mice deficient in the MGAT5 gene display an increased susceptibility to autoimmune diseases (7, 10, 11). These mice lacking GnT-V function (no synthesis of β1,6-GlcNAc branched N-glycan structures) display an increased TCR clustering and increased T helper 1 (Th1) differentiation that result in a hyperimmune response in mouse models of multiple sclerosis (7, 10). Interestingly, in mouse models of experimental autoimmune encephalomyelitis (EAE) and type 1 diabetes, it was shown that supplementation with GlcNAc induces increased N-glycan branching, through increasing the hexosamine pathway, that was associated with inhibition of T cell growth and differentiation (12, 13), leading to delayed disease progression. Moreover, N-glycan branching was also found to regulate T cell development (14). Recently, it was demonstrated that

Significance

Our findings demonstrate that metabolic supplementation of mucosal T cells, isolated from patients with active ulcerative colitis (UC), with N-acetylglucosamine (GlcNAc) leads to the enhancement of branched N-glycosylation on the T cell receptor, which was associated with the control of T cell activation and function. These results were validated in “glycoengineered” mouse models with severe colitis. Overall, our results open new avenues for a target-specific therapy in inflammatory bowel disease (IBD). The therapeutic use of GlcNAc (either alone or in combination with other antifibrotic therapies) represents a simple immunomodulatory strategy in IBD, with absence of side effects, low costs, and the possibility of being used as a simple rescue therapy to avoid unnecessary toxic effects and step-up therapies in IBD.


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branching N-glycans potentiate the differentiation of induced regulatory (iTreg) T cells over Th17 differentiation (15). Importantly, and in the setting of human immune-mediated disorders, we have recently found that patients with UC exhibit a deficiency in branched glycosylation (catalyzed by N-glycan branching enzyme) on mucosal T cells that was associated with disease severity. Patients with UC who have severe disease showed the most pronounced defect on branched N-glycans in intestinal T cells, together with a significant reduction of MGAT5 gene transcription in these cells (16).

In the present study, and building upon our previous findings in patients with UC (16), we have evaluated the impact of glycosylation, particularly the branched N-glycosylation pathway, in the regulation of the T cell-mediated immune response in patients with UC. We further explored whether this mechanism could be therapeutically targeted in vivo through a simple glycan-based strategy. Our results showed that metabolic supplementation of mucosal T cells, isolated from patients with active UC, with GlcNAc led to the enhancement of branched N-glycosylation on the TCR, controlling T cell activation and function. Preclinical data further demonstrated that GlcNAc treatment of MGAT5 null or heterozygous mice developing severe forms of induced colitis significantly controlled disease severity and progression due to suppression of the intestinal T cell-mediated immune response, protecting gut epithelial cells where GlcNAc was topically administered by gavage. Altogether, this study highlights the potential of glycans as novel immunomodulatory agents in IBD, warranting validation in human clinical trials.

Results
Ex Vivo GlcNAc Supplementation Increased Branched N-Glycosylation of T Cells from Patients with Active UC. We have previously demonstrated that patients with UC display reduced branched N-glycosylation on mucosal T cells (16). To assess the ability of glycans as reparers of the above-mentioned mechanistic defect, we herein promoted, ex vivo, the hexosamine biosynthetic pathway (SI Appendix, Fig. S4) by providing intestinal T cells with GlcNAc supplementation with GlcNAc. Previous studies showed that supplementation with GlcNAc increases the availability of the substrate (UDP-GlcNAc) to Golgi enzymes such as GnT-V, enhancing β1,6-GlcNAc branching N-glycans, particularly in T cells (13). To test this hypothesis, T cells (CD3+/CD8+) were isolated ex vivo from both the intestinal lamina propria of fresh colonic biopsies and peripheral blood of patients with UC who have active disease and were supplemented with increasing doses of GlcNAc. Different GlcNAc concentrations (40 mM, 80 mM, and 100 mM) were tested, and 100 mM was further employed for the following experiments (SI Appendix, Fig. S1B). The expression of β1,6-GlcNAc branched N-glycans on colonic T cells was evaluated by flow cytometry using Phasolus vulgaris leukoagglutinating (L-PHA) lectin. We observed a dose-dependent increase of branched N-glycans on intestinal T cells upon GlcNAc supplementation across different patients (Fig. 1A). This increased modification with branched N-glycans was also observed in T cells isolated from peripheral blood mononuclear cells of patients with active UC displaying FSC-high and SSC-high light-scattering parameters, characteristic of activated lymphocytes (Fig. 1B). The increased expression of β1,6-GlcNAc branched N-glycans was detected both on CD4+ and CD8+ T cells (SI Appendix, Fig. S1 C and D). No effects of GlcNAc treatment in the proportion of CD4+ and CD8+ T cell subsets in the cultures were observed (SI Appendix, Fig. S1 E and F), supporting that GlcNAc supplementation leads to a specific modification with branched glycans on T cells in a dose-dependent manner. Importantly, the enhancement of branched N-glycans was only observed in T cells from patients with active UC (Fig. 1C and SI Appendix, Fig. S1G). T cells from healthy controls and from patients with inactive disease did not show alterations in the levels of branched glycans upon treatment with increasing concentrations of GlcNAc (Fig. 1C and SI Appendix, Fig. S2), possibly due to the higher baseline branching comparing with patients with active UC. These results were further confirmed by other technical approaches. Increased expression of β1,6-GlcNAc branched N-glycans on a band the same size as the TCR β-chain (TCRβ) after GlcNAc supplementation was also detected by L-PHA bloting (Fig. 1C) and by TCR immunoprecipitation using lysates of lamina propria T lymphocytes (LPLs) purified from patients with UC (Fig. 1D). Interestingly, this increased branching of N-glycans after GlcNAc supplementation was found to occur in intestinal T cells from patients with UC with different Mayo subscres (with Mayo endoscopic subscores 1, 2, and 3) with a trend association with disease severity, as depicted in SI Appendix, Fig. SIG. The internalization of externally given GlcNAc was already demonstrated in cell lines (17). The specific effects of GlcNAc in enhancing branched glycosylation on T cells from patients with active UC was further demonstrated by the reversed effects on L-PHA mean fluorescence intensity when T cells were treated with the N-glycan branching inhibitors kifunensine (KF) and swainsonine (SW) (SI Appendix, Fig. S3) in T cells from biopsies and blood of patients with active UC. Moreover, the specific effects of GlcNAc in the enhancement of branched glycosylation in T cells was further validated by supplementation of T cells from patients with active UC with other glycan types such as α-mannose, which revealed no impact in branched N-glycan expression (SI Appendix, Fig. S3). To further validate these observations, we also performed imaging flow cytometry showing that TCRβ+ T cells display an increase of fluorescence intensity due to staining with L-PHA on the cell membrane. This increase was observed in T cells displaying blast-like morphology (Fig. 1E and E1). Taken together, these results demonstrate that metabolic supplementation with active UC with GlcNAc promotes the hexosamine biosynthetic pathway enhancing β1,6-GlcNAc branched N-glycans on the TCR, and thus restoring the deficiency on branched N-glycans previously shown in mucosal T cells from patients with UC (16). Next, we have determined the specificity of this enhancement of β1,6-GlcNAc N-glycan branching by analyzing the correspondent GnT-V enzymatic activity. Interestingly, and in line with our previous observations on MGAT5 gene transcription (16), T cells from patients with active UC displayed reduced GnT-V enzymatic activity compared with healthy controls (SI Appendix, Fig. S4). Our results showed that the increased GlcNAc enzymatic activity of T cells could be significantly recovered after metabolic supplementation with GlcNAc (Fig. 2A), which further supports the effects of GlcNAc in the enhancement of N-glycan branching mediated by GnT-V. In the N-glycosylation branching pathway, the β1,6-GlcNAc branched N-glycan catalyzed by GnT-V can be further extended with polylactosamine structures (ligands for galectins), which, in turn, can be terminally sialylated (SI Appendix, Fig. S4). Our results showed that GlcNAc supplementation of ex vivo activated T cells led to increased expression of β1,6-GlcNAc branched N-glycans (as detected by L-PHA lectin) (Fig. 1E and 2B and B1) with a trend of increased expression compared with untreated T cells (Fig. 2B and B1). Additionally, we determined whether there was a terminal addition of α2,6-linked sialic acid, recognized by binding of Sambucus nigra agglutinin (SNA), and/or α2,3-sialic acid, recognized by Maackia amurensis agglutinin (MAL-II). The results demonstrated a trend of increase in α2,6-linked sialic acid, and no consistent alterations in α2,3-sialic acid linkages were detected (Fig. 2B and B1). Overall, our results support that ex vivo supplementation of T cells from patients with UC with GlcNAc has the potential to enhance the branched N-glycosylation on T cells, remodeling the T cell glycoprofile, which is ultimately expected to have an impact on the regulation of the immune response.

Shaping the T Cell-Mediated Immune Response in UC Through Increased Branching N-Glycans. After demonstrating the ability of GlcNAc supplementation to repair the deficiency of branched N-glycans on ex vivo T cells, we next evaluated its impact on the modulation of T cell responsiveness. The metabolic supplementation with GlcNAc of ex vivo activated T cells from naive patients (without therapy) resulted in significant suppression of their proliferative response to anti-CD3/CD28 mAb stimulation (Fig. 3 A and A1). In addition, and importantly, the increased N-glycan branching on T cells resulted in a significant reduction in the production of the proinflammatory cytokines TNF-α, IFN-γ, and IL-17A, which are associated with UC pathogenesis (18),
in a GlcNAc dose-dependent manner (Fig. 3B), supporting its effect on the control of Th1/Th17-type immune responses. The effects of GlcNAc on the suppression of proinflammatory cytokine production were found to be independent of cell death and/or decreased T cell proliferation. After normalization of cytokine concentrations to the respective T cell proliferation index (index of division), we still observed a reduction in the production of proinflammatory cytokines (SI Appendix, Fig. S5A). Moreover, the percentage of IFN-γ- and TNF-α-expressing cells among total CD4+ T cells, evaluated by intracellular flow cytometry, was decreased upon GlcNAc treatment (Fig. 3B). The impact of GlcNAc supplementation on other cytokines, such as TGF-β and IL-10, is not significant (SI Appendix, Fig. S5 B and C). The specific effects of GlcNAc in suppressing proinflammatory cytokine production is further confirmed by inhibitors (KF and SW), which blocked the regulatory impact of branched N-glycans in T cell function (SI Appendix, Fig. S6). Next, we have evaluated the expression of the transcription factors (T-bet, RORγt, Foxp3, and Gata3) in T cells (CD3+ ) under ex vivo GlcNAc treatment. The results showed that GlcNAc supplementation was associated with a reduction in the expression of T-bet and RORγt, which corroborates the negative impact on the Th1/Th17-type response (Fig. 3 C and CI). The expression of Foxp3, a transcription factor associated with regulatory T cells as well as with human activated T cells (18), was also found to be reduced upon GlcNAc treatment. To gain insight into the molecular basis of this modulation of the T cell response through metabolic supplementation with GlcNAc, we analyzed its impact on the TCR signaling pathway. We observed that GlcNAc supplementation led to an inhibition of the phosphorylation of ZAP70 and LAT, thereby hindering T cell activity by controlling the TCR signaling pathway (Fig. 3D). Additionally, we evaluated whether GlcNAc supplementation had an impact on T cell apoptosis. Our results showed that T cells from patients with active UC treated with GlcNAc displayed an increased susceptibility to apoptosis compared with nontreated T lymphocytes as soon as 3 h after stimulation (SI Appendix, Fig. S7A). This effect was not seen in T cells from controls and patients with inactive UC, which demonstrated no differences, or even a trend to decrease apoptosis, respectively, at the same time points upon GlcNAc supplementation. This argues against a putative effect of hyperosmolarity in the observed increase of apoptosis/cell death in GlcNAc-supplemented T cells from patients with active UC and in controls (16) (SI Appendix, Fig. S7A). Treatment with the branched N-glycan inhibitors (KF and SW) did not reverse the effects of GlcNAc in apoptosis (SI Appendix, Fig. S7B). At 72 h, a significant decrease in branched glycosylation levels was observed with KF and SW (SI Appendix, Fig. S3); however, no differences in the apoptosis were detected (SI Appendix, Fig. S7B). The percentage of cell death was unchanged or even higher with SW and KF than with treatment with GlcNAc only (SI Appendix, Fig. S7B1). Taken together, these data collectively demonstrate that ex vivo GlcNAc supplementation of T cells from patients with active UC enhances the branched N-glycans and resulted in a significant suppression of T cell proliferation and TCR signaling, as well as controlled Th1/Th17-type immune responses.

**Treatment with GlcNAc Reduces Disease Severity and Ameliorates Clinical Signs of Disease in Mice with Colitis.** To determine whether dysregulation of branched N-glycans on TCR occurs in different experimental mouse models of colitis, we have evaluated two different chemically induced colitis mouse models, the dextran sodium sulfate (DSS)-induced and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced models (19), in C57BL/6 wild-type mice. In both models, colitis was successfully induced, and we have observed a dysregulation of branched N-glycans on TCR (SI Appendix, Fig. S8 A and B), which is in accordance with our previous observations on human patients with UC (16). The DSS-induced colitis model was selected as the ideal one to proceed with, since the rectal induction of disease in the TNBS model would interfere with the cecum administration of GlcNAc. After disease

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**Fig. 1.** Ex vivo GlcNAc supplementation of T cells from patients with UC resulted in increased branching N-glycans. (A and B) CD3+ T cells from patients with active UC were cultured with different concentrations (millimolar) of GlcNAc, and the fold change of mean fluorescence intensity due to L-PHA staining was determined by flow cytometry. The scatter plots illustrate the mean ± SEM of five biological replicates. One-way ANOVA using the Newman–Keuls multiple comparison posttest: **P ≤ 0.05; ***P ≤ 0.01; ****P ≤ 0.001. (C) Protein lysates of purified CD3+ T cells under GlcNAc treatment were subjected to L-PHA lectin blotting to evaluate the expression levels of p1,6-GlcNAc branched N-glycans on a protein band corresponding to the migration profile of the TCR. WB, Western blot. (Inset) Linear regression using mean values per treatment condition. (D) Immunoprecipitation (IP) of TCR followed by β1,6-GlcNAc branched N-glycan recognition with L-PHA. The density of bands is indicated below each band. (E) Imaging flow cytometry analysis (on an ImageStreamX) of L-PHA membrane distribution on TCR+ cells after GlcNAc supplementation in T cells isolated from blood of patients with active UC. Representative images of activated T cells display blast-like morphology showing colocalization (overlaid images) of TCR and L-PHA. The density of bands is indicated below each band. (Fig. 1B) Immunoprecipitation (IP) of TCR followed by β1,6-GlcNAc branched N-glycan recognition with L-PHA. The density of bands is indicated below each band. (E) Imaging flow cytometry analysis (on an ImageStreamX) of L-PHA membrane distribution on TCR+ cells after GlcNAc supplementation in T cells isolated from blood of patients with active UC. Representative images of activated T cells display blast-like morphology showing colocalization (overlaid images) of TCR and L-PHA. The density of bands is indicated below each band.
Fig. 2. Remodeling of the glycosylation phenotype upon metabolic supplementation with GlcNAc. (A) Impact of GlcNAc supplementation on GntV activity was determined using a pool of lysates from treated vs. nontreated peripheral blood T cells, in three biological replicates of patients with active UC, from two independent technical experiments. Student’s t test: **p ≤ 0.01. (B) Flow cytometry evaluation of glycophenotype of T cells upon GlcNAc supplementation. (B1) Scatter plot: fold change of MFI due to staining with each lectin on T cells, in two biological replicates with different stages of disease severity (Mayo endoscopic scores 1 and 2), from two independent experiments. Results are normalized to the untreated condition, which was taken as 1. Student’s t test: *p ≤ 0.05. NS, not significant.

Deficiency in Branched Glycosylation in MGAT5 Null or Heterozygous Mice Is Associated with Early-Onset Disease and Increased Severity of Colitis That Is Suppressed by GlcNAc Treatment. To gain insights into the immuno-phenotype effects of MGAT5 on Th17 cell activity and signaling. Interestingly, LPLs from GlcNAc-treated mice displayed a decrease in the phosphorylation levels of ZAP70 and LAT that was more pronounced in mice treated with GlcNAc enemas (Fig. 5B). This result supports control of T cell activity through GlcNAc treatment, with effective results in topical/enema administration. Next, the effects of GlcNAc administration on the expression levels of T-bet, the transcription factor associated with proinflammatory Th1 cell polarization, were assessed in situ in the intestinal lamina propria. We have observed abundant cells expressing T-bet in lymphocytic infiltrates in mice with colitis that were markedly reduced in GlcNAc-treated mice (Fig. 5B, Insets). Furthermore, mice treated with GlcNAc revealed a significant reduction of IFN-γ production and a trend in the suppression of IL-17A induction, further supporting that the enhancement of branched N-glycans by GlcNAc treatment controls Th1/Th17-type immune responses in vivo (Fig. 5E). Taken together, our in vivo results demonstrate a therapeutic effect of GlcNAc in a colitis-induced mouse model, revealing the immunomodulatory properties of this agent in the control of intestinal inflammation and, consequently, in the control of disease severity and progression.

onset using 2% DSS, GlcNAc was administered through two different routes: orally, by supplementing the drinking water with 0.25 mg/mL GlcNAc, and/or rectally, using 0.5 mg/mL GlcNAc enemas. With this approach, we assessed the therapeutic effects of GlcNAc on disease activity and on the control of intestinal inflammation. The experiment design is summarized in Fig. 4A. Our results showed that mice with colitis and treated with GlcNAc exhibited lower body weight loss (Fig. 4B) and significant improvements in disease activity index (DAI) (Fig. 4C) in comparison to mice with colitis not treated with GlcNAc (DSS control). Importantly, whenever animals received GlcNAc topically by enema administration (either alone or in combination with oral GlcNAc), the body weight changes were lower in comparison to animals receiving only oral GlcNAc. These results suggest that GlcNAc enemas may have promising topical effects on the control of disease severity (Fig. 4B and C, Insets). Accordingly, macroscopic observation of the colon showed that mice with colitis displayed visible colonic edema (swelling of the bowel wall) that recovered upon GlcNAc treatment (SI Appendix, Fig. S8C). In addition, mice with colitis displayed extensive lymphocytic infiltrates (Fig. 4D, arrowhead) presenting with CD3+ cells (Fig. 5A) in the intestinal lamina propria, together with notable alterations of the glandular morphology. Furthermore, these animals also showed a reduced mucus layer (produced by glycoproteins at the glycocalyx), a natural barrier that confers protection from microbiota, preventing disease aggressiveness and progression (20). When animals with colitis were treated with GlcNAc, there was a decrease in the lymphocytic infiltrate and an improvement of the glandular architecture (Fig. 4D), which is compatible with disease remission (21). To further investigate the relationship between this overall improvement of disease severity through GlcNAc treatment and the levels of β1,6-GlcNAc branched N-glycans on LPLs, we performed L-PHA and CD3 histochemistry in the formalin-fixed, paraffin-embedded (FFPE) colonic specimens from the different groups of animals (Fig. 5A). The results showed that induction of colitis was accompanied by a notable intestinal lymphocytic infiltrate in the lamina propria, including CD3-expressing cells that expressed low levels of branched N-glycans. When mice were treated with GlcNAc, an increased expression of β1,6-branched glycans in the inflammatory infiltrate was observed (Fig. 5A, arrowheads). Importantly, the administration of GlcNAc also resulted in increased mucus lining, with augmented expression of branched glycans in glycoproteins in the superficial mucus layer, which is compatible with disease remission. Afterward, we investigated whether the TCR was particularly targeted by the observed enhancement of branched N-glycans in vivo. The results showed that GlcNAc treatment resulted in increased branching glycosylation on intestinal T cell, particularly in the TCR (Fig. 5B and B1). To further address the mechanistic basis of the clinical efficacy of GlcNAc, we have evaluated the impact on T cell activity and signaling. Interestingly, LPLs from GlcNAc-treated mice displayed a decrease in the phosphorylation levels of ZAP70 and LAT that was more pronounced in mice treated with GlcNAc enemas (Fig. 5C). This result supports control of T cell activity through GlcNAc treatment, with effective results in topical/enema administration. Next, the effects of GlcNAc administration on the expression levels of T-bet, the transcription factor associated with proinflammatory Th1 cell polarization, were assessed in situ in the intestinal lamina propria. We have observed abundant cells expressing T-bet in lymphocytic infiltrates in mice with colitis that were markedly reduced in GlcNAc-treated mice (Fig. 5B, Insets). Furthermore, mice treated with GlcNAc revealed a significant reduction of IFN-γ production and a trend in the suppression of IL-17A induction, further supporting that the enhancement of branched N-glycans by GlcNAc treatment controls Th1/Th17-type immune responses in vivo (Fig. 5E). Taken together, our in vivo results demonstrate a therapeutic effect of GlcNAc in a colitis-induced mouse model, revealing the immunomodulatory properties of this agent in the control of intestinal inflammation and, consequently, in the control of disease severity and progression.
were also less responsive to GlcNAc, which makes GlcNAc therapy in these mice more challenging. Nevertheless, the results in MGAT5<sup>−/−</sup> and MGAT5<sup>+/-</sup> mice (Fig. 6 B–D) showed that even in these mice, treatment with GlcNAc improved clinical scores compared with the scores of DSS mice. This beneficial effect was pronounced whenever GlcNAc was administered topically by enema (single or in combination with oral administration), as evidenced by lower body weight loss and lower colitis scores compared with DSS mice. Interestingly, when GlcNAc treatment was initiated earlier in disease course of MGAT5<sup>−/−</sup> mice, at day 5, when animals started to develop severe colitis, we observed a decrease of the colitis scores compared with non-treated diseased animals (DSS), which supports the benefits of treating these susceptible animals earlier with GlcNAc, as demonstrated in Fig. 6D.

**GlcNAc Treatment of MGAT5 Null or Heterozygous Mice Attenuates Disease Progression by Controlling Th1/Th17-Type Immune Responses.**

MGAT5 null and heterozygous mice showed disorganization of the glandular architecture and an increase of inflammatory infiltrates in the colonic mucosa after DSS-induced colitis that was improved overall upon GlcNAc treatment (Fig. 7A). Notably, the evaluation of β1,6-GlcNAc branched N-glycan levels on CD3<sup>+</sup> LPLs confirmed that the induction of colitis was accompanied by decreased expression of branched N-glycans in LPLs from MGAT5<sup>−/−</sup> mice and by the absence of branched N-glycans in MGAT5<sup>+<//-</sup> mice with induced colitis (DSS) (Fig. 7A). Interestingly, when mice of both MGAT5 genotypes were treated with GlcNAc, a recovery of β1,6-branched glycan expression was observed in the intestinal inflammatory infiltrate (Fig. 7A, arrowhead) and in the superficial mucus layer, which is compatible with mucosal healing. The positive detection of L-PHA staining in MGAT5<sup>−/−</sup> null mice upon GlcNAc supplementation was unexpected, as these mice lack the GnT-V enzyme. This positive detection was confirmed at the protein level by L-PHA blot (Fig. 7B and SI Appendix, Fig. S9A). In fact, the reactivity of L-PHA in MGAT5<sup>−/−</sup> mice is the lowest, compared with heterozygous and WT mice, but it is still positive. These observations may be in line with redundant effects of other GnTs at the Golgi (22–24) that, within an activated hexosamine pathway, may compensate for the absence of MGAT5 by producing the β1,6-GlcNAc branched glycan, although with a much lower yield of synthesis, as we have observed. In an attempt to explore the potential compensatory synthesis of β1,6-GlcNAc branched N-glycans in MGAT5<sup>−/−</sup> null mice, we have observed that the MGAT5<sup>b</sup> gene [a homologous gene of MGAT5A that codifies the GnT-IX or GnT-Vb enzyme (25)] is apparently up-regulated in colonic T cells from MGAT5<sup>−/−</sup> null mice upon GlcNAc treatment. This preliminary evidence suggests GnT-IX/Vb as a potential candidate that might compensate for the synthesis of β1,6-GlcNAc branched glycan in MGAT5<sup>−/−</sup> null mice. This issue needs further investigation. We then assessed the enhancement of branched N-glycans specifically on T cells after GlcNAc treatment. MGAT5<sup>−/−</sup> null or heterozygous mice treated with GlcNAc showed an enhanced expression of branched N-glycans in the TCR compared with nontreated diseased animals (DSS) (Fig. 7 C and D). This effect was highlighted when animals were treated topically with GlcNAc enemas (Fig. 7 C and D). To explore the mechanistic effects of GlcNAc treatment in the T cell-mediated immune response, the
Impact on TCR signaling was evaluated in MGAT5 null and heterozygous mice. An overall decrease of ZAP70 phosphorylation, indicative of reduced TCR signaling, was detected in colonic T cells from GlcNAc-treated mice that was evident when GlcNAc was administered topically (Fig. 7E). This topical effect was particularly observed in MGAT5<sup>−/−</sup> mice, where oral treatment did not affect TCR signaling. The more marked effect achieved through GlcNAc enema administration suggests that this molecule may be more efficiently taken up by cells in this way, likely by increasing its local concentration, thus facilitating its entry into the hexosamine pathway and usage by glycosyltransferases other than GnT-V (SI Appendix, Fig. S9A) that may redundantly catalyze the branched N-glycans. These redundant effects need further investigation. Additionally, the evaluation of the Th1 proinflammatory response revealed that GlcNAc treatment in both genotypes was associated with reduced proportions of cells expressing T-bet in lymphocytic infiltrates compared with control mice with colitis (Fig. 7F). Notably, similar to our observations of GlcNAc treatment in T cells from patients with UC, colonic explants from MGAT5 null or heterozygous mice treated with GlcNAc indicate a trend for IFN-γ expression, but with a more pronounced effect in reducing IL-17A.

These results further support the impact of GlcNAc treatment and, consequently, the enhancement of branched N-glycans in controlling Th1/Th17-type immune responses also in the IBD in vivo model (Fig. 7G). Regarding the impact of GlcNAc on regulatory T cells, no apparent difference in the numbers of FoxP3-expressing cells was observed at the intestinal lamina propria comparing non-GlcNAc-treated mice of MGAT5<sup>wt</sup> and MGAT5<sup>−/−</sup> genotypes (SI Appendix, Fig. S9C). Nevertheless, further studies are needed to better characterize the regulatory effects of GlcNAc treatment in the different components of the immune response, such as in macrophages (SI Appendix, Fig. S9 D and E), as proposed by previous reports (26), and other T cell populations. Taken together, these data support the targeted-specific effects of GlcNAc that were able to repair the deficiency in branched glycosylation on T cells associated with MGAT5 deficiency, thus controlling progression of colitis.

Discussion

IBD is characterized by a substantial heterogeneity concerning disease onset, course, response to therapy, and progression to complications (e.g., hospitalization, need for surgery, cancer) (1). Moreover, and despite recent advances in IBD therapeutic resources, a high proportion of patients remain refractory to conventional treatment, and approximately half of the patients with UC do not achieve...
sustained remission (27). In addition, issues related to side effects and failure in therapy response highlight the need for more effective and target-specific drugs (28). We have recently demonstrated that patients with UC exhibit a deficiency in branched glycosylation on intestinal T cells due to a transcriptional reduction of the MGAT5 gene that accompanied disease severity (16).

Herein, we uncovered a prominent role for the branched glycosylation pathway in IBD pathogenesis, by shaping the course of the T cell response. This pathway is thus an attractive target for novel therapies. Indeed, we have shown here that checkpoints of T cell immune response in UC could be modulated by metabolic supplementation with the simple sugar GlcNAc. We showed that GlcNAc therapy concomitantly increased branched N-glycosylation on T cells and down-regulated T cell proinflammatory responses both ex vivo and in vivo. In line with these observations, it was previously reported that lack of β1,6-GlcNAc branched N-glycans, by targeted deletion of the locus encoding GntV, results in enhanced TCR signaling and increased susceptibility to multiple sclerosis (7, 10). Moreover, and in accordance with our results, the increase of N-glycan branching through GlcNAc salvage into the hexosamine pathway was associated with a decreased threshold in T cell activation and more stable CTLA-4 surface expression, which resulted in the inhibition of adoptively transferred EAE (7, 13).

The evidence presented here suggests that GlcNAc supplementation of T cells isolated from patients with active UC resulted in remodeling of the glycoophenotype of T lymphocytes through a marked increase of β1,6-GlcNAc branched N-glycans and an increase of polylactosamine structures, the ligand for galectins (29), that can then be terminally sialylated, predominantly with α2,6-sialic acid residues. This glycan reprogramming on T cells was shown to translate into key immunomodulatory effects in UC. Importantly, the enhancement of branched N-glycans on T cells induced by GlcNAc supplementation led to the suppression of T cell proliferation; inhibition of T cell signaling; reduced production of the proinflammatory cytokines TNF-α, INF-γ, and IL-17A; and controlled Th1- and Th17-type responses. Both Th responses have been associated with IBD pathogenesis (30). Furthermore, these results are in accordance with a very recent report using mouse T cell cultures, which showed the ability of GlcNAc to promote iTreg over Th17 differentiation (15).

The glycosylation of T cells can also have an impact on the susceptibility to cell death (31). Accordingly, treatment of T cells with GlcNAc induced an increased susceptibility to apoptosis, which is at the core of different regulatory processes controlling T cell activation and avoiding pathological inflammation (32). This effect of GlcNAc on apoptosis was dose-dependent and limited to T cells from patients with active UC (SI Appendix, Fig. S7B). In agreement, previous reports have shown that extension with polylactosamine structures, which are ligands for galectins (such as galectin 1), was associated with proapoptotic effects of CD4+ T cells (31, 33). Nevertheless, and given that treatment with KF and SW did not reverse the apoptotic effects induced by GlcNAc in the cells of patients with active UC, it cannot be excluded that factors other than branching glycans can also contribute to GlcNAc-mediated regulation of T cells apoptosis. Importantly, besides the TCR, the enhancement of branched N-glycosylation can also modify other receptors like the coreceptors CD4 and CD8, as well as the growth inhibitory receptor CTLA-4 (7, 10, 22). Moreover, CD45 and CD25 are also potential targets of branched glycosylation modification that can further contribute to the regulation of the T cell-mediated immune response through branching N-glycans (15, 34). Immunomodulation through GlcNAc-mediated enhancement of branched glycosylation, as described here, is a promising therapeutic approach to restore T cell homeostasis in IBD (SI Appendix, Fig. S10). Indeed, metabolic regulation of T cell function has been highlighted by recent research (35) and may be manipulated to reduce T cell-mediated inflammation (15, 36).

The preclinical data reported here provide the proof of concept supporting such a therapeutic approach in IBD. Deficiency of the MGAT5 gene was associated with higher susceptibility to severe forms of colitis and early-onset disease. These data highlight the prominent role of branched N-glycosylation in the pathogenesis of IBD, and are in accordance with previous studies on multiple sclerosis (11). GlcNAc supplementation improved clinical scores and was associated with a better disease course in mice developing the most severe disease phenotype (MGAT5+−/−). These immune-suppressive effects catalyzed by GlcNAc were observed by both oral and enema administration routes, with promising effects when mice received GlcNAc topicaly via enemas. This topical effect of GlcNAc is in line with the ability of GlcNAc to be more efficiently taken up by the intestinal mucosa, thereby entering directly into the
hexosamine pathway to increase branched glycosylation. Intriguingly, in MGAT5−/−, with the absence of MGAT5/GnT-V, expression of the β1,6-GlcNAc branched N-glycans detected by L-PHA lectin histochemistry and blotting was positive and slightly increased with GlcNAc treatment. This unexpected result may be in accordance with the fact that several glycosyltransferase-deficient mice exhibit no or only mild phenotypes due to redundancy or compensation of glycan functions. In fact, several family members of glycosyltransferases are known to be functionally redundant (37, 38). This potential redundant effect observed in MGAT5−/− mice treated with GlcNAc might be due to the fact that other Golgi glycosyltransferases within an activated hexosamine pathway triggered by UDP-GlcNAc may compensate for the lack of this specific glycan structure, thereby guaranteeing immune homeostasis. One of the candidate enzymes is the homologous GnT-Vb that may compensate for the synthesis of the branched glycans; however, further studies are needed to clarify this issue. Similar examples of the redundancy of glycosyltransferases were described, such as for FUT8-deficient mice (39). Nevertheless, this redundant effect observed upon GlcNAc supplementation needs to be further explored.

The clinical effects of GlcNAc were further evidenced by the decreased frequency of T-bet−expressing cells in colonic mucosa of treated mice, together with reduced release of the proinflammatory cytokine IL-17A of respective colonic explants. GlcNAc is a naturally occurring amino sugar for which no adverse effects were reported in humans (40). It is available as a dietary supplement, and oral GlcNAc administration showed no toxicity in rats (41). Interestingly, 17 y ago, oral GlcNAc was described to promote intestinal lining through mucus production in children with severe treatment-resistant IBD (21). In that study, eight of the 12 children studied went into clinical remission, which could have resulted from the immunomodulatory effects of GlcNAc.
Isolation of CD3^+ T Cells from Fresh Colonic Biopsies and Blood of Patients with Active UC. Ex Vivo Culture of T Cells. Dias et al. PNAS

Flow cytometry. CD3^+ T cells (from biopsy and blood) were stained with an EasySep Human T Cell Enrichment Kit (STEMCELL Technologies) following the manufacturer’s instructions. CD3^+ T cells were cultured for 72 h with anti-CD3 mAb (clone OKT3) and soluble anti-CD28 mAb (clone CD28.2) (eBioscience). T cell cultures were supplemented with GlcNAc (Sigma and Wellesley Therapeutics, Inc.).

Imaging Flow Cytometry. Imaging flow cytometry analysis was performed as previously described (42).

Flow Cytometry. CD3^+ T cells were stained with CD4 and CD8 (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated L-PHA (Vector Laboratories), as well as with cell surface markers (CD4, CD45), intracellular antigens (T-bet, and fluorescein isothiocyanate (FITC)-conjugated L-PHA (Vector Laboratories), were supplemented with GlcNAc (Sigma and Wellesley Therapeutics, Inc.).

Materials and Methods

Patient Selection and Colonic Biopsy Collection. Fresh colonic biopsies were obtained from three patients in remission diagnose with UC and normal controls (n = 3) who underwent a scheduled colonoscopy (2014–2017) at the Gastroenterology Department of Centro Hospitalar do Porto–Hospital do Santo António (CHP-HSA), Porto, Portugal. All participants gave informed consent, and procedures were approved by the Ethics Committee of CHP/HSA [233/12/179-DEFI/177-CESJ].

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Cytokine Production. Supernatants from colonic T cells were stained with 5-(and-6)-carboxyfluorescein diacetae succinimidyl ester (CFSE) using a CellTrace CFSE Cell Proliferation Kit (Invitrogen), as described by Oliveira et al. (43).

Cytokine Production. Supernatants from colonic T cell cultures were analyzed by flow cytometry using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) following the manufacturer’s instructions. Human TGF-β1 quantification was performed using ELISA kits (R&D Systems) according to the manufacturer’s instructions. The supernatants from mouse colonic explant cultures were concentrated using Amicon Ultra-2 10 kDa Centrifugal Filters (Merck Millipore), according to manufacturer’s instructions. The levels of IFN-γ and IL-17A (anti-mouse, Ready-SET-Go! kits; eBioscience), and TNF-α and IL-6 (anti-mouse; Biologend) were quantified by ELISA, according to the manufacturer’s instructions.

Western Blot and TCR Signaling. TCR signaling and L-PHA lectin blot analysis (44), using T cell protein lysates (extracted with radioimmunoprecipitation buffer), were performed as described by Dias et al. (16). Incubation of phospho-Zap-70 (Tyr191),phospho-p38 MAPK, phospho-JNK, and phospho-ERK kinases, with T cells was performed as previously described. Goat anti-rabbit IgG-HRP antibody, and rabbit IgG antiactin (Santa Cruz Biotechnology) or mouse IgG antitubulin (Sigma) was used as a loading control.

Immunoprecipitation. TCR immunoprecipitation, using total cell lysates obtained from mouse colons or from ex vivo human T cell cultures, was performed as previously described (16).

Glycophenotype. T cells were incubated with biotinylated L-PHA, biotinylated LEI, biotinylated SNL, or biotinylated MAL-II (Vector Labs). Lectins were revealed with FITC-conjugated streptavidin.

Apoptosis Assays. Apoptotic cells were identified by flow cytometry, using an FITC Annexin V ApoTox Detection Kit I (BD Biosciences), following the manufacturer’s instructions.

Enzymatic Reaction and HPLC Analysis. The Gnt-V enzymatic activity analyses in T cells from patients with UC and controls were performed as previously described by Takamatsu et al. (45).

DSS- and TNBS-Induced Colitis and in Vivo GlcNAc Treatment. Colitis was induced with DSS in C57BL/6, MGATS wild-type, heterozygous, and knockout mice (kindly provided by Michael Pierce, University of Georgia, Athens, GA) (19). The TNBS model was also performed using C57BL/6 mice (19). After disease onset, DSS mice were treated with GlcNAc (Sigma and Wellesley Therapeutics, Inc.) (12). LPLs were isolated from mouse colon as previously described (16).

Tissue Immunohistochemistry and Immunofluorescence. FFPE colonic tissue slides were used for H&E staining and for immunohistochemistry with L-PHA and anti-CD3 mAb as well as FoXP3 and F4/80, as described, respectively, by Dias et al. (16) and Teixeira et al. (46). For T-bet immunofluorescence, mouse IgG, T-bet-specific mAb (clone 4810; Santa Cruz Biotechnology), goat anti-mouse Alexa 594 secondary antibody (Invitrogen), and DAPI staining were used.

Real-Time PCR. Total RNA from isolated LPLs was extracted and the quantitative real-time PCR (qRT-PCR) was performed using TaqMan Gene Expression Assays (Applied Biosystems), as previously described (16). qRT-PCR was carried out in triplicates for the target gene MGAT5b (Taqman probe: Mm01252571_m1) and the endogenous control 18S (Hs99999901_s1) (Applied Biosystems) (16).

Statistics. Statistical significance was assessed by one-way or two-way ANOVA test and, where appropriate, by an unpaired Student’s t test (two-tailed) using GraphPad Prism 5. P values of <0.05 were considered statistically significant.

More details can be found in SI Appendix, SI Materials and Methods.

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