Glucosamine Modulates T Cell Differentiation through Down-regulating N-Linked Glycosylation of CD25*

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Background: Glucosamine is an amino sugar that has immunoregulatory effects on T cell-mediated diseases. However, the mechanism(s) through which glucosamine modulates different T cell subsets and diseases remain unclear. We demonstrate that glucosamine impedes Th1, Th2, and Treg but promotes Th17 differentiation through down-regulating N-linked glycosylation of CD25 and subsequently inhibiting its downstream Stat5 signaling in a dose-dependent manner. The effect of glucosamine on T helper cell differentiation was similar to that induced by anti-IL-2 treatment, further supporting an IL-2 signaling-dependent modulation. Interestingly, excess glucose rescued this glucosamine-mediated regulation, suggesting a functional competition between glucose and glucosamine. High-dose glucosamine significantly decreased Glut1 N-glycosylation in Th1-polarized cells. This finding suggests that both down-regulated IL-2 signaling and Glut1-dependent glycolytic metabolism contribute to the inhibition of Th1 differentiation by glucosamine. Finally, glucosamine treatment inhibited Th1 cells in vivo, prolonged the survival of islet grafts in diabetic recipients, and exacerbated the severity of EAE. Taken together, our results indicate that glucosamine interferes with N-glycosylation of CD25, and thereby attenuates IL-2 downstream signaling. These effects suggest that glucosamine may be an important modulator of T cell differentiation and immune homeostasis.

Results: Glucosamine inhibits Th1, Th2, iTreg cells, but promotes Th17 cell development through interference with N-glycosylation of CD25.

Conclusion: Glucosamine modulates T cell differentiation in vivo and subsequently influences the progression and severity of autoimmune diseases.

Significance: Glucosamine-mediated modulation of CD25 glycosylation can be beneficial to controlling autoimmune diseases.

Glucosamine has immunomodulatory effects on autoimmune diseases. However, the mechanism(s) through which glucosamine modulates different T cell subsets and diseases remain unclear. We demonstrate that glucosamine impedes Th1, Th2, and iTreg but promotes Th17 differentiation through down-regulating N-linked glycosylation of CD25 and subsequently inhibiting its downstream Stat5 signaling in a dose-dependent manner. The effect of glucosamine on T helper cell differentiation was similar to that induced by anti-IL-2 treatment, further supporting an IL-2 signaling-dependent modulation. Interestingly, excess glucose rescued this glucosamine-mediated regulation, suggesting a functional competition between glucose and glucosamine. High-dose glucosamine significantly decreased Glut1 N-glycosylation in Th1-polarized cells. This finding suggests that both down-regulated IL-2 signaling and Glut1-dependent glycolytic metabolism contribute to the inhibition of Th1 differentiation by glucosamine. Finally, glucosamine treatment inhibited Th1 cells in vivo, prolonged the survival of islet grafts in diabetic recipients, and exacerbated the severity of EAE. Taken together, our results indicate that glucosamine interferes with N-glycosylation of CD25, and thereby attenuates IL-2 downstream signaling. These effects suggest that glucosamine may be an important modulator of T cell differentiation and immune homeostasis.

After encountering antigens, naïve CD4 T cells are activated and differentiated into effector subsets such as Th1, Th2, Th17, and Treg cells. Environmental cytokines such as IFN-γ, IL-4, IL-6, IL-12, and TGF-β play central roles in determining the differentiation fate of these cells (1–3). IL-2, an important cytokine produced mainly by activated CD4 T cells, is also critically involved in this process. IL-2Rβ (CD122) and γc (CD132) are both responsible for signaling transduction. IL-2Rα (CD25), which is expressed mainly on activated T cells or Treg cells (2, 4, 5), also plays a major role in the high-affinity binding to IL-2 in conjunction with IL-2Rβγc. This binding activates three major pathways, MAPK, PI3K/Akt/mTOR, and signal transducer and activator of transcription 5 (Stat5)2 to regulate T cell survival (6), proliferation (7–9), differentiation (10–12), and activation activation-induced cell death (13).

It has been reported that IL-2 signaling increases the expression of the IL-12Rβ2, T-bet, IL-4Rα, and Gata-3 but suppresses the expression of IL-6Rα, IL-6 signal transducer gp130, and RoRγt in Stat5-dependent manner, which leads to stronger Th1 and Th2 differentiation and weaker Th17 differentiation (10, 12, 14). IL-2-mediated Stat5 activation also promotes Foxp3 expression to induce Treg cell differentiation (15).

Glucosamine, an amino sugar, exerts immunosuppressive effects on IL-2-mediated T cell proliferation and cytokine secretion in the mixed-lymphocyte reaction (MLR) (16, 17). Glucosamine negatively regulates both Th1/Th17-mediated autoimmune encephalomyelitis (EAE) and Th2-dominant atopic dermatitis in vivo (18, 19). Considering the functional similarity and/or overlapping effects on T cell differentiation between glucosamine and IL-2 blockage, we hypothesized that glucosamine may modulate T helper cell-mediated autoimmune diseases by interfering with IL-2-signaling. However, the detailed mechanism(s) underlying the effects of glucosamine may be an important modulator of T cell differentiation and immune homeostasis.

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2 The abbreviations used are: Stat, signal transducer and activator of transcription; HBP, hexosamine biosynthetic pathway; EAE, autoimmune encephalomyelitis; PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylidenamino) N-phenylcarbamate; BADGP, benzyl 2-acetamido-2-deoxy-beta-D-galactopyranoside.

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on IL-2-mediated T helper cell differentiation still are incompletely understood.

Glucosamine has been reported to interfere in the processing of N-linked glycosylation (20, 21), suggesting a pivotal role at the interface of signaling. N-Linked glycosylation is a ubiquitous post-translational modification of secretory proteins such as fetuin and antibodies, and of membrane-anchored receptors such as epidermal growth factor receptor (EGFR). N-Linked glycosylation is crucial for the folding, stability, and function of these glycoproteins. N-Glycosylation can be modulated in a nutrient-responsive manner through the hexosamine biosynthetic pathway (HBP). This pathway is a branch of glucose metabolism to produce UDP-GlcNAc, which is subsequently used in N-glycosylation and O-GlcNAcylation, and this pathway is sensitive to the availability of glucose, glutamine, and acetyl-CoA (22). On the other hand, the N-acetylglucosaminyltransferase (Mgat5), one of the Golgi N-acetylglucosaminyltransferases, transfers GlcNAc from UDP-GlcNAc to generate tetra-antennary N-glycan in a nutrient-responsive manner (23, 24). Mgat5-mediated N-glycan branching has been reported to regulate positively the threshold of TCR activation (23, 25) and the stability of cytokine receptors (26). Previous study suggested that glucose availability would attenuate the threshold of TCR activation (23, 25) and the stability of cytokine receptors (26). Previous study suggested that glucose availability would attenuate the threshold of TCR activation (23, 25) and the stability of cytokine receptors (26).

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**Experimental Procedures**

**Reagents**—Glucosamine HCl (G4875), 2-deoxy-d-glucose (D6134), tunicamycin (T7765), O-(2-amidino-2-deoxy-d-glucopyranosyldenedimino) N-phenylcarbamate (PUGNAC, OGA inhibitor), benzyl 2-acetamido-2-deoxy-α-d-galactopyranoside (BADGP, OGT inhibitor) were purchased from Sigma-Aldrich.

**Mice**—NOD/Sybr (K^d, D^b, I-A^k, I-E^null) and BDC2.5 transgenic mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). NOD/SCID and C57BL/6 mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) and subsequently bred in the specific pathogen-free facility of the animal center in the National Defense Medical Center (Taipei, Taiwan), which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care International.

**T Cell Differentiation**—Naive CD4 T cells isolated from NOD mice by AutoMACS were stimulated with plate-coated anti-CD3 (5 μg/ml) plus soluble anti-CD28 (1 μg/ml) monoclonal antibodies (mAbs) under Th1 (murine IL-12, 10 ng/ml; anti-murine IL-4, 10 μg/ml), Th2 (murine IL-4, 20 ng/ml; anti-murine IL-12p40, 5 μg/ml; anti-murine IFN-γ, 10 μg/ml), Th17 (human TGF-β, 5 ng/ml; murine IL-6, 50 ng/ml; anti-murine IFN-γ, 10 μg/ml; anti-murine IL-4, 10 μg/ml), or iTreg (human TGF-β, 5 ng/ml; human IL-2, 10 ng/ml) condition for 3 days. Where stated, cultures were supplemented with anti-murine IL-2 antibodies (clones S4B6 and JES6–1A12 from ebioscience), anti-murine CD25 antibodies (clones 3C7 and PC61 from Biologend). For intracellular staining, cells were restimulated with PMA and ionomycin (Sigma-Aldrich) in the presence of monesin (Sigma-Aldrich) for 4 h.

**Flow Cytometric Analysis**—Lymphocytes were stained with fluorochrome-conjugated Abs specific for murine CD4 (RM4–5), IFN-γ (XMG1.2), IL-17A (eBio17B7), IL-2 (JES6–5H4), and Foxp3 (FJK-16s), which were purchased from ebioscience (San Diego, CA). Fluorochrome-conjugated Abs to murine IL-4 (11B11), CD25 (PC61), CD69 (H1.2F3), CD122 (TM-β1), and CD132 (PC61), which were purchased from BioLegend (San Diego, CA). Intracellular phospho-Stats staining was performed on differentiated T helper cells. Cells were harvested and fixed in Fixation buffer for 30 min at 4 °C and then permeabilized with perm buffer III (BD Pharmingen, San Jose, CA) on ice for 30 min. Cells were stained with phospho-Stat3 Y705, phospho-Stat4 Y693, phospho-Stat5 Y694, and phospho-Stat6 Y641 for 60 min at room temperature. Secreted cytokines were assessed by the mouse FlowCytomix Multiplex bead assay (Affymetrix) according to the manufacturer’s instructions. Cells and multiplex beads were analyzed using FACS-Calibur flow cytometry (BD Pharmingen).

**Western Blot Analysis**—After stimulation, cells were immediately resuspended in lysis buffer (50 mM Tris, pH 7.4% 2% SDS, 150 mM NaCl) containing protease inhibitor mixture (Sigma) and/or phosphatase inhibitor mixture (Roche, Mannheim, Germany). Cell lysates were analyzed by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with Abs to phospho-AktT^473, Akt (Cell Signaling Technology), CD25, Stat5 (Santa Cruz), phospho-Stat5 Y^694, phospho-ERK1/2 Y^202/204, ERK1, (Abcam, Cambridge, UK), O-GlcNAc (CTD110.6; Covance, Berkeley, CA), and β-actin (AC-15 clone, Sigma-Aldrich).

**Protein Deglycosylation of Whole Cell Lysates**—The denaturing cell lysates were deglycosylated with PNGase F only or Proteins Deglycosylation Mix (containing PNGase F, sialidase A, O-glycosidase, β-N-acetylglucosaminidase, and β1–4galactosidase) according to the manufacturer’s instructions (NEB).

**Quantitative RT-PCR**—RNA was isolated (Qiagen, Valencia, CA) and used for cDNA synthesis with the SuperScript III syn-
thesis kit (Invitrogen, Carlsbad, CA). An ABI StepOnePlus Real-time PCR system was used for quantitative PCR, with primers and SYBR Green probe sets (Roche, Mannheim, Germany). The expression of target gene is presented as the fold change relative to the expression of control samples. Primer sequences used to determine expression of T-helper-cell-related transcriptional factors or other target genes were T-bet (Forward, 5'-CGTGTG TGGGAAGCTGAGAG-3'; Reverse, 5'-CCACA TCCACAAACATCCTG-3'), GATA-3 (Forward, 5'-AGAACCGGCCCTTTGAAA-3'; Reverse, 5'-AGTTCC CGCAGGATGGTCC-3'), RoRγt (Forward, 5'-ACCTCCACTG CCAAGCTGTTGCTGTC-3'; Reverse, 5'-CATTCTGCA TCTCGCATGTA GCTGCCC-3'), Foxp3 (Forward, 5'-GGCCCTTCT CCAGGACAGA-3'; Reverse, 5'-GCTGTG CATG GCTGGGTGTT-3'), or Rps29 (Forward, 5'-ACG GT CTGATCCGCAATAC-3'; Reverse, 5'-AGCA TGATC GGTCACTTG-3')

Adaptive Transfer—CD4⁺CD25⁺ BDC2.5 T cells were enriched by using autoMACS (Miltenyi Biotec) and 1 × 10⁶ cells were transferred into 6–8-week-old female NOD/SCID mice via the retro-orbital plexus. Recipient mice were treated intraperitoneally daily with glucosamine (2 g/kg body weight) or solvent alone (PBS). Diabetes of NOD/SCID recipients was defined as blood glucose >300 mg/dL at two consecutive tests. Different lymphoid organs from the recipient mice were harvested 8 days following adoptive transfer and the percentage of IFN-γ or IL-4 by Th1 or Th2 cells, respectively, and markedly increased the production of IL-17A by Th17 cells (Fig. 1B), indicating that glucosamine modulates different T helper cell differentiation in vitro.

T cell differentiation is orchestrated by cooperative induction of cytokines and transcription factors to facilitate the development of specific lineages. We next investigated whether glucosamine modulates the expression of transcriptional factors during T cell polarization. As expected, glucosamine treatment inhibited the expression of T-bet, Gata-3, and Foxp3 in Th1-, Th2-, and iTreg-polarized cells, respectively. Interestingly, glucosamine administration only modestly increased RORγt expression in Th17-polarized cells (Fig. 1C). This subtle change in RORγt expression found here could not fully explain the significant promotion of IL-17A in these glucosamine-treated cells. Nevertheless, our findings suggest that glucosamine systemically modulates T helper cell differentiation through the regulation of transcriptional factors. To exclude the possibility that these glucosamine-mediated effects on the differentiation of distinct subsets are dependent on T cell proliferation, we polarized CD4 T cells under the Th1 or Th17 condition with CFSE labeling for 72 h in the presence of glucosamine (Fig. 1D). The cell division of the Th1 or Th17 subset treated with glucosamine was similar to that of untreated cells, suggesting that glucosamine-mediated inhibition of Th1, Th2, and iTreg, and promotion of Th17 cells is proliferation independent.

Glucosamine Impairs IL-2-mediated Phosphorylation of Stat5 through Down-regulation of IL-2Ra Surface Expression—Cytokine signaling regulates the expression of T cell lineage-specific transcriptional factors and contributes to T helper cell differentiation through the activation of Stats. We next evaluated the effect of glucosamine on the phosphorylation of Stats under different T cell-polarizing conditions. Glucosamine slightly inhibited Stat4, Stat6, and Stat3 phosphorylation in Th1-, Th2-, and Th17-polarized cells, respectively (Fig. 2A). These small changes in phosphorylation levels, especially the down-regulation of Stat3 phosphorylation were not able to support the significant decrease in the effector cytokines IFN-γ and
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IL-4 and the marked increase in IL-17A in cells incubated with glucosamine. The IL-2-dependent activation of Stat5 has been reported to play an important role in the regulation of effector and regulatory T cell differentiation (10–12, 14, 29). We investigated whether Stat5 phosphorylation is modulated by glucosamine and whether this contributes to T helper cell development. Glucosamine markedly inhibited Stat5 phosphorylation under different T cell-polarizing conditions (Fig. 2B). These results are consistent with previous reports and explain, at least in part, how glucosamine-mediated down-regulation of activated Stat5 contributes to the inhibition of Th1, Th2, and iTreg and promotion of Th17 cells (11, 12, 30, 31).

To evaluate further whether diminished p-Stat5-mediated inhibition of Th1, Th2, and iTreg cells, and promotion of Th17 cells is IL-2 signaling dependent, we analyzed T helper cell development in the presence of neutralizing anti-IL2 antibody. Th1-, Th2-, Th17-, or iTreg-polarizing cells treated with anti-IL-2 antibody displayed differentiation patterns similar to those observed in cells incubated with glucosamine, supporting the idea that the effects of glucosamine on T helper cell differentiation are IL-2 signaling dependent (Fig. 2C). The p-Stat5 levels in anti-IL-2 antibody-treated cells were similar to those in glucosamine-incubated cells (data not shown). This finding confirmed that modulation by glucosamine of the development of T helper cells is IL-2-Stat5 axis dependent. To investigate further whether glucosamine can affect the expression level of IL-2R and subsequently can cause the down-regulation of p-Stat5, we used flow cytometry to analyze three subunits of the IL-2R: IL-2Rα (CD25), IL-2Rβ, and γ chain. Glucosamine significantly inhibited the expression of IL-2Rα on T cells (Fig. 2D) but had minimal effects on IL-2Rβ and γc in these cells (Fig. 2D). Next, to evaluate whether the inhibitory effect of glucosamine on CD25 is blocked by high-dose exogenous IL-2, we analyzed T cell polarization in the presence of high-dose exogenous IL-2 and glucosamine. Our results revealed that high-dose exogenous IL-2 (100–1000 IU/ml) does not interfere with T cell development under glucosamine treatment (Fig. 2E). To further clarify whether the blockade of CD25 responds differently to glucosamine-mediated effect, we analyzed T helper polarization in the presence of anti-CD25 antibody and glucosamine. Results from flow cytometric analysis indicated that Th1, Th2, and iTreg cells were significantly inhibited and Th17 cells were markedly promoted by this CD25 blockade, consistent with the results observed in glucosamine-treated cells (Fig. 2E). Finally, the polarization patterns of T cells under a combined treatment of glucosamine and anti-CD25 antibody were very similar to those observed in glucosamine- or anti-CD25 antibody-treated group (Fig. 2E), implying that T cells with or without CD25 blockade/deficiency respond similarity to glucosamine during different polarization conditions. These results further support the idea that glucosamine modulates T helper subset development through the attenuation of IL-2Rα surface expression.

Effect of Glucosamine on T Helper Cell Polarization is O-GlcNAcylation Independent—Glucosamine is taken up into cells by glucose transporters and is converted mainly to UDP-GlcNAc, the starting material for the serine/threonine-based O-GlcNAcylation of intracellular proteins (32, 33). We measured the O-GlcNAcylation levels in CD4 T cells treated with glucosamine. As expected, both glucosamine and PUGNAc (O-GlcNAcase inhibitor) increased the O-GlcNAcylation level, and BAADGP (O-GlcNAc transferase inhibitor) moderately attenuated its level in activated CD4 cells (Fig. 3A)

Previous studies have shown that the O-GlcNAc modifications of NF-AT and NF-κB stimulate T cell activation and cytokine production (34, 35). We investigated whether an increase in O-GlcNAcylation can modulate T helper cell differentiation. To mimic the effect of glucosamine in increasing O-GlcNAcylation level, we analyzed T helper cell polarization in the presence of PUGNAc. Consistent with the findings of a previous report (34), CD69 expression increased in PUGNAc-treated cells. A similar increase in CD69 expression was also observed in glucosamine-treated cells (Fig. 3B). However, compared with measurements in PBS-treated cells, PUGNAc-stimulated O-GlcNAcylation did not affect CD25 expression or T helper cell development (Fig. 3, C and D). These results imply that the effects of glucosamine on CD25 expression and T helper cell polarization do not occur directly through O-GlcNAcylation-based regulation.

Glucosamine Attenuates IL-2Rα Surface Retention on CD4 T Cells by Interfering with N-linked Glycosylation—In addition to the ability to increase O-GlcNAcylation, glucosamine has been reported to affect N-linked glycosylation (36). It has also been shown that post-translational N-linked glycosylation regulates the signaling pathway by maintaining the stability of surface cytokine receptors (26, 37). Because CD25 is a highly glycosylated molecule, we postulated that glucosamine attenuates CD25 expression on the surface of CD4 T cells by interfering with its N-linked glycosylation. Western blot analysis showed clearly that the expression of IL-2-signaling molecules, including CD25, p-Stat5 and p-Akt, was significantly up-regulated after T cell activation and that this effect was time dependent (Fig. 4A). Interestingly, glucosamine treatment significantly attenuated the expression level of CD25, the p-Stat5 and p-Akt in a time-dependent and dose-dependent manner (Fig. 4, A and B). This glucosamine-mediated down-regulation of IL-2 signaling molecules is consistent with the results observed in cells treated with neutralizing anti-IL-2 antibodies, and confirms that the effects of glucosamine on T helper cell differentiation is IL-2 signaling dependent (Fig. 2C). Interestingly, the molecular mass of CD25 was markedly decreased in glucosamine-treated CD4 T cells, suggesting that the glycosylated status of CD25

FIGURE 1. Modulatory effect of glucosamine during different T helper cell differentiation. A, naive T cells were polarized under different T helper cell conditions with indicated concentration of glucosamine for 3 days. Representative flow cytometry and frequency of IFN-γ, IL-4, IL-17A, and Foxp3 on CD4 T cells from the indicated conditions with the results of six independent experiments plotted in the right panel. B, cytokine productions in the culture supernatant collected from the indicated conditions were determined by FlowCytomix Multiplex bead assay (n = 3/group). C, quantitative RT-PCR was performed to analyze gene expression of Tbox21, Gata-3, Rorc, or Foxp3 mRNA in Th1, Th2, Th17, or iTreg-polarized cells for 2 days, respectively (n = 3/group). D, CFSE-labeled CD4 T cells were cultured with Th1 and Th17 conditions with glucosamine for 3 days. Representative flow cytometry and cell division of IFN-γ or IL-17A on CD4 T cells with results of three independent experiments plotted in the right panel. *, p < 0.05; **, p < 0.01.
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A

Th1  Th2  Th17

Unstimulated  Polarized + PBS  Polarized + GlcN

- - -

Th1  Th2  Th17

Unstimulated  Polarized + PBS  Polarized + GlcN

- - -

B

Th1  Th2  Th17  iTreg

Unstimulated  Polarized + PBS  Polarized + GlcN

- - -

C

PBS  GlcN  PBS  GlcN

anti-IL-2 Abs

IFN-γ  IL-4  IL-17  Foxp3

D

E

IL-2 (IU/ml)

Polarized + PBS  Polarized + GlcN

- 10  100  1000

PBS  GlcN  GlcN  GlcN  GlcN  αCD25  αCD25 + GlcN

Th1  Th2  Th17  iTreg

αCD25  αCD25 + GlcN

- 10  100  1000

PBS  GlcN  GlcN  GlcN  GlcN  αCD25  αCD25 + GlcN

Th1  Th2  Th17  iTreg

αCD25  αCD25 + GlcN

- 10  100  1000
was highly modulated by glucosamine (Fig. 4, A and B). To further confirm whether the inhibitory effect of glucosamine on CD25 is blocked by high-dose exogenous IL-2, we treated high-dose exogenous IL-2 in glucosamine-treated cells. Our results revealed that high-dose exogenous IL-2 (100 to 1000 IU/ml) does not restore the glucosamine-mediated down-regulation of p-Stat5 and p-Akt (Fig. 4C). Moreover, we analyzed T cell activation in the presence of anti-CD25 antibody, which mimicking the condition of CD25 blockage. Results from Western blotting analysis indicated that clearly the p-Stat5 and p-Akt were significantly decreased under anti-CD25 antibody treatment, similar to glucosamine-mediated effect, consistent with the results observed in glucosamine-treated cells (Fig. 4C), further suggesting that the effect of glucosamine on T helper cell differentiation is IL-2/CD25 dependent.

To evaluate whether the reduced molecular mass of CD25 in glucosamine-treated CD4 T cells occurred through interference with N-linked glycosylation, we analyzed its molecular mass in CD4 T cells in the presence of tunicamycin, a specific inhibitor of N-linked glycosylation. Interestingly, the molecular mass and expression level of CD25 were significantly decreased, and the p-Stat5 and p-Akt were also diminished; these patterns were almost identical to those observed in cells treated with glucosamine (Fig. 4D). These data support the idea that the...
Glucosamine-mediated decrease in CD25 molecular mass is N-linked glycosylation dependent. To confirm the effect of glucosamine on the N-glycosylated modification of CD25, we treated CD4 T cell lysates with PNGase F, an enzyme that removes N-glycan. Western blot analysis showed clearly that the molecule mass of CD25 in PNGase F-treated cell lysates was lower than that in untreated cell lysates (Fig. 4E). The molecular mass of CD25 in PNGase F-treated cell lysates was similar to that observed in glucosamine-treated cells, suggesting that CD25 contains N-glycosylated modification and that glucosamine-mediated decrease in molecular mass occurs through interference with the N-glycosylated modification. To investigate fur-
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A

| Glc (mM) | 19 | 19 |
|----------|----|----|
| GlcN (mM)| 5  | 5  |

IB: CD25
IB: p-Stat5 (Y694)
IB: Stat5
IB: p-Akt (S473)
IB: Akt
IB: p-ERK1/2 (Thr202/Tyr204)
IB: ERK1
IB: Actin

B

PBS
Glc 19 mM
GlcN
Glc+GlcN

$CD25$

Glc (mM) 0 19 19
GlcN

GlcN

C

| Glc (mM) | 19 | 19 |
|----------|----|----|
| Th1
| IFN-γ |
| 58.4 |
| 58 |
| 30.2 |
| 40 |

Th2

| IL-4 |
| 14.6 |
| 9.08 |
| 1.29 |
| 3.68 |

Th17

| IL-17A |
| 6.35 |
| 6.91 |
| 41 |
| 16 |

iTReg

| IL-17A |
| 0.41 |
| 0.5 |
| 7 |
| 2.8 |

Foxp3

| Foxp3 |
| 69 |
| 61 |
| 42 |
| 52.4 |
ther whether O-linked glycosylation also contributes to the modification of CD25, we treated CD4 T cell lysates with a combination of five deglycosylating enzymes (PNGase F, sialidase A, O-glycosidase, β-N-acetylgalcosaminidase and β1,4galactosidase). The combined enzymes-treated cell lysates showed the lowest molecular mass of CD25 compared with the PNGase F-treated or glucosamine-incubated lysates (Fig. 4E). These data suggest that after combination enzymes treatment, the size further decreased and therefore implying for the presence of simple O-glycans on CD25 that can be removed and that the glucosamine-mediated suppression of glycosylation of CD25 is mainly N-linked glycosylation dependent. We next investigated whether tunicamycin has glucosamine-like modulatory effects on T cell differentiation. Tunicamycin significantly inhibited Th1, Th2, and iTreg cells and increased Th17 polarization (Fig. 4F). These results suggested that glucosamine-mediated down-regulation of N-linked glycosylation of CD25 and the subsequent decrease in IL-2 signaling play a critical role in modulating the differentiation of CD4 T cells.

Excess-free Glucose Restores Glucosamine-mediated Suppression of N-linked Glycosylation of CD25—It has been reported that glucose is required for N-linked glycosylation of growth factor receptors and the subsequent regulation of cell growth and survival (37). Because glucosamine is structurally similar to glucose and is taken up into cells by the glucose transporters (38), we hypothesized that the modulatory effect of glucosamine on N-linked glycosylation occurs through competitive inhibition of the glucose transport. To test this hypothesis, we treated CD4 T lymphocytes with a higher concentration of glucose (19 mM) in the presence of glucosamine. Interestingly, treatment with excess glucose partially rescued the glucosamine-mediated down-regulation of N-linked glycosylation of CD25 and p-Stat5 level (Fig. 5A). This result implies that the glucosamine-mediated effect on N-linked glycosylation occurs through interference in glucose uptake/metabolism. Moreover, flow cytometry showed that excess glucose partially restored the surface expression of CD25 in glucosamine-treated CD4 T cells (Fig. 5B), supporting the idea that glucosamine-mediated inhibitory effects on N-linked glycosylation of CD25 occurs through interference with glucose uptake/metabolism.

We next investigated whether glucosamine-modulated CD4 T cell differentiation can be restored by excess glucose. A higher glucose concentration significantly rescued the glucosamine-mediated effects on T helper cell differentiation (Fig. 5C), indicating the functional competition between glucosamine and glucose in T helper cell development.

**Glucosamine Down-regulates Glut1 Expression and the Glycolytic Pathway by Interfering with N-linked Glycosylation in Polarized Th1 Cells—**Several studies have reported that the glycolytic pathway plays a critical role in effector T helper cell differentiation and function (39, 40). Our results showed clearly that a population of Th1 cells was significantly inhibited by glucosamine at higher concentrations such as 5 or 7.5 mM compared with cells treated with a low concentration of glucosamine (0.1-2.5 mM) or PBS (Fig. 1A). Considering these findings, we hypothesized that a high concentration of glucosamine (5 or 7.5 mM) would downregulate Th1 cell development by interfering with the glycolytic pathway. To test this hypothesis, we first evaluated whether glucosamine could affect glucose uptake and lactate production during Th1 polarization. Glucose uptake and lactate production were significantly decreased in the late phase of culture (48–72 h) at 5 mM glucosamine, but not in the early phase (within 24 h) (Fig. 6, A and B). This result implies that the effects of glucosamine on glucose uptake and lactate production do not occur through an immediate and/or direct competition with glucose transporters. Glucosamine at a low concentration (2.5 mM) did not interfere with the glycolytic pathway during Th1 polarization, and significant inhibition was observed only at 5 mM or higher (Fig. 6, C and D). Our results support the idea that both the glycolytic pathway and the IL-2-Stat5 axis are involved in Th1 differentiation.

Glut1 is markedly expressed on activated T cells and effector T helper subsets such as Th1, Th2, and Th17 cells (40), and is a highly N-glycosylated molecule that is involved in the glycolytic pathway (41, 42). We hypothesized that glucosamine modulates Glut1-associated glucose uptake and lactate production through the blockade of the N-linked glycosylation of Glut1. Western blotting analysis showed that Glut1 expression was markedly up-regulated at 48–72 h during Th1 differentiation (Fig. 6, E, left panel). The level of glucose uptake correlated positively with lactate production at 48–72 h and the expression of Glut1 during Th1 cell differentiation. This finding supports the idea that Glut1 expression plays a critical role in modulating effector T cell development. Interestingly, glucosamine treatment markedly reduced the molecular mass of Glut1 in a dose-dependent manner (Fig. 6, E and F, left panels). To evaluate further whether the decrease in molecular mass of Glut1 in glucosamine-treated Th1 cells occurs through the down-regulation of its N-linked glycosylation, we compared the Glut1 molecular pattern between glucosamine- and PNGase F-treated cell lysates. Western blotting analysis showed a low molecular mass of Glut1 in glucosamine-treated lysates (Fig. 6, E and F), which was similar to that of PNGase F-treated cell lysates (Fig. 6, E and F, right panels). This finding supports the idea that the glucosamine-mediated decrease in the molecular mass of Glut1 occurs through interference with the N-glicosylated modification.

Finally, we investigated whether glucosamine-mediated down-regulation of N-linked glycosylation of Glut1 could modulate the glycolytic pathway and inhibit Th1 development. Tunicamycin-mediated blockade of the N-linked glycosylation of Glut1 and inhibition of glucose uptake and lactate production (Fig. 6, G–J) consequently interfered with Th1 polarization (Fig. 4E). These findings further suggest that glucosamine

![FIGURE 5. Excess glucose rescues glucosamine-mediated suppression of N-linked glycosylation.](image-url)}
inhibits glucose uptake and/or its metabolism through down-regulating N-linked glycosylation of Glut1, instead of competing against glucose for glucose transporters. Moreover, the glucosamine-mediated down-regulation of Th1 cells occurs at least partly through the N-glycosylation-defective Glut1-mediated inhibition of glucose uptake and lactate production. Our
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data also indicate that the glucosamine-mediated down-regulation of N-linked glycosylation of CD25 and Glut1 critically modulates Th1 cell differentiation.

Glucosamine Prevents the Progression of Autoimmune Diabetes and Exacerbates the Severity of EAE through the Modulation of Th1 and Th17 Cell Differentiation in Vivo—As noted above, we found that glucosamine significantly inhibited Th1, Th2, and Treg cell differentiation, and promoted Th17 cell development in vitro (Fig. 1A). Glucosamine treatment has been reported to modulate inflammation in EAE (18) and atopic dermatitis (19). However, the potential influence of glucosamine on CD4 T cell development and autoimmune diabetogenesis have not been examined in vivo. We thus evaluated whether glucosamine could suppress Th1-mediated autoimmune diabetes in the NOD model (43, 44). We adoptively transferred CD4⁺CD25⁻ T cells from BDC 2.5 NOD mice which express a transgenic TCR with specificity for the islet antigen chromogranin A, into NOD/SCID mice (3). The mice were treated daily with glucosamine (60 mg/day/mouse) or vehicle control. Five of the eight control mice (62.5%) became diabetic by day 8 and all were diabetic by day 9 after transfer (Fig. 7A). By contrast, the diabetic kinetics in glucosamine-treated mice was slowed significantly (p < 0.001), demonstrating a protective effect of glucosamine against this Th1-mediated autoimmune diabetes. Histological analysis revealed more intact (grade 0) and low-infiltrated (grade 1) islets in the glucosamine-treated recipients compared with PBS-injected controls (Fig. 7B), supporting the idea that glucosamine treatment in vivo attenuated the development of the disease by attenuating the diabetogenic properties of lymphocytes. The pathogenic T cells in the pancreas of NOD mice are mainly IFN-γ-producing cells (45). We next investigated whether glucosamine treatment could modulate the Th1 development in the recipient mice. The absolute numbers of IFN-γ-producing CD4 T cells in pancreatic lymph nodes (PLNs) and in pancreata were significantly lower in glucosamine-treated mice than in PBS-injected controls (Fig. 7C). Studies have demonstrated that the priming of diabeticogenic T cells occurs in PLNs before the migration of these T cells into the pancreas (46, 47). Our results are consistent with this observation in that treatment of glucosamine appeared to suppress Th1 cell differentiation in PLNs and to subsequently decrease their infiltration into pancreatic islets. To investigate further whether glucosamine treatment could prolong the survival of islet grafts in diabetic NOD recipients, we isolated islets from young (6–8-week-old) male NOD mice and implanted them into the left kidney capsule of newly diabetic recipients treated daily with glucosamine (10 mg/mouse) or vehicle. The mean survival time of islet grafts was much longer in the glucosamine-treated mice than in PBS-treated mice (24.5 versus 7 days, p < 0.001; Fig. 7D), demonstrating that glucosamine treatment significantly protected the islet grafts from autoimmune attack. Our findings suggest that glucosamine-mediated down-regulation of IFN-γ-producing CD4 T cells delayed the onset of diabetes after BDC 2.5 T cell transfer and prolonged the survival of islet grafts in diabetic recipients. A previous study has indicated that Th1/Th17-mediated EAE was significantly suppressed when glucosamine was administrated at 10 mg/day/mouse i.p (18). Although low concentration of glucosamine (1–2.5 mM) does not influence Th1 cell differentiation, it significantly promotes Th17 cell development in vitro (Fig. 1A). Therefore we investigated whether low dosage of glucosamine (10 mg/every other day/mouse) treatment could modulate the induction and severity of EAE (48, 49). We injected glucosamine intraperitoneally into mice with induce EAE and monitored the disease kinetics. The onset of disease was 2 days earlier in glucosamine-treated mice than that in PBS-injected mice (day 7 versus day 9), and the clinical manifestations of EAE were more exacerbated in the glucosamine-treated mice (p < 0.001; Fig. 7E). Histological analysis showed a significant lymphocyte infiltration into the spinal cord at day 8 in the glucosamine-treated group (Fig. 7F), a finding that is consistent with their disease process and severity. To investigate further whether this exacerbated phenotype was related to an increase in the percentages of Th17 cells induced by glucosamine, we evaluated the percentages of Th17 cells in the spleen, draining lymph nodes, and CNS of glucosamine-treated mice on day 8 after induction. We found a higher frequency of Th17 cells in the CNS but not in the draining lymph nodes and spleen of glucosamine-treated mice compared with the PBS-treated controls (Fig. 7G). This finding supports the idea that glucosamine promotes Th17 cell generation in vivo and subsequently stimulates the progression of EAE. Taken together, our results demonstrate that glucosamine systemically modulates Th1 and Th17 cell differentiation in vivo and subsequently influences the progression and severity of autoimmune diseases.

Discussion

In this study, our results demonstrate that glucosamine-mediated inhibition of N-linked glycosylation of CD25 and the subsequent decrease in IL-2 signaling play critical roles in modulating the differentiation of CD4 T cells and the development of autoimmune diseases. We also found that tunicamycin has glucosamine-like modulatory effects on IL-2-mediated T helper cell differentiation, supporting the idea that inhibition of N-linked glycosylation on CD25 modulates IL-2-mediated T helper cell development. Consistent with these in vitro findings, glucosamine treatment significantly modulated Th1 and Th17 cell development in vivo and influenced the progression and severity of autoimmune diabetes and EAE.
In our study, we observed that glucosamine slightly attenuated the phosphorylation of Stat3, and significantly increased Th17 development (Fig. 2A). Our results also showed that the glucosamine-mediated down-regulation of CD25 occurs through interference with its N-linked glycosylation (Fig. 4A). This finding is similar to that of a previous report showing that
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N-linked glycosylation of gp130, a highly glycosylated molecule, is significantly decreased in human prostate carcinoma DU145 cells treated with glucosamine (50). Moreover, a recent study demonstrated that the inhibitory effect of IL-2 on Th17 cell differentiation is mainly Stat5 dependent and not RORγt or Foxp3 dependent (51). These results may explain why, although glucosamine slightly decreases Stat3 phosphorylation during Th17 cell polarization, inhibition of Stat5 activation induced by glucosamine apparently overrides this Stat3-mediated effect and results in a significant promotion of Th17 cells, implying a dominant role of the IL-2-Stat5 axis during T helper cell differentiation.

It has been reported that inhibition of N-linked glycosylation by glucosamine alters the molecular mass of Cox-2 in A549 cells (36), and gp130 in DU145 cells (50). Consistent with previous studies, our results revealed that glucosamine treatment significantly decreased the molecular mass of CD25 on activated T cells through interference with N-linked glycosylation (Fig. 4, A and B). Interestingly, although O-linked glycosylation is mediated, at least in part, by the depletion of intracellular nucleotide pools and that glucosamine transport is competitively inhibited by glucose in Novikoff rat hepatoma cells (38). Consistent with this report, our results revealed that treatment with excess glucose partially restored the modulatory effects of glucosamine on N-linked glycosylation of CD25 and IL-2-dependent T helper cell differentiation (Fig. 5, A and C), suggesting that glucose and glucosamine have competitive effects, probably via glucose transporters. On the other hand, several reports have demonstrated that glucosamine significantly increases the levels of O-GlcNAcylation in different cell types (33, 53, 54). Consistent with these previous findings, our results have indeed revealed that glucosamine increases the levels of O-GlcNAcylation in CD4 T cells (Fig. 3A), and yet, at the same time, inhibits N-glycosylation of CD25. It suggested that a supply of exogenous glucosamine may have more complicated and/or cell-type dependent effect than simply increasing a common intracellular pool of UDP-GlcNAc.

IFN-γ produced by Th1 cells contributes to the pathogenesis of autoimmune diabetes, and blockage of IFN-γ delays the onset of diabetes in NOD mice (44, 55). Consistent with these earlier reports, our results indicated that glucosamine treatment inhibited Th1 cell differentiation and suppressed diabetogenesis in NOD/SCID mice that had received adoptive transfer of BDC2.5 CD4 T cells (Fig. 7, A and C). The number of pathogenic T cells in pancreatic lymph nodes and pancreas was significantly decreased in the recipient mice (data not shown). Integrin, a highly glycosylated molecule, is involved in cell adhesion and migration, and the blockage of integrin protects NOD mice from the development of the diabetes (56). We therefore propose that glucosamine treatment may also interfere with N-linked glycosylation of these adhesion molecules and may thus attenuate the migration of pathogenic T cells into pancreatic lymph nodes and the pancreas.

Several reports have demonstrated that impaired N-glycosylation in Mgat5-deficient mice exacerbates EAE incidence and severity by multiple mechanisms including lower T cell activation threshold and surface retention of CTLA4 (22, 23, 57, 58). Lack of Mgat5 has also been reported to decrease cytokine signaling and, consequently, to influence cell functions by increasing the endocytosis of cytokine receptors (26). These results imply that defective N-glycosylation of cytokine receptors and the TCR or costimulatory molecules such as CTLA4 on T cells disturbs the immune tolerance and contributes to the development of autoimmune diseases. Consistent with these reports, our results revealed that inhibition of N-glycosylation of CD25 by glucosamine significantly promoted Th17 cell differentiation and accelerated the development of EAE. Our findings also support the idea that impairment of the IL-2–CD25–Stat5 axis promotes Th17 development, which is consistent with previous studies demonstrating an inhibitory effect of IL-2 on Th17 cell differentiation in vitro and in vivo (51, 59, 60). By contrast, a previous report showed that glucosamine attenuated the functions of T cells and microglia/macrophages and attenuated the progression of EAE (18). These differences in the effects of glucosamine on EAE induction and severity between these two studies may reflect differences in glucosamine dosage and/or the complex experimental approaches.

In summary, although glucosamine increases the O-GlcNAc modification of proteins during T cell activation, our results indicate that glucosamine may interfere with N-glycosylation of CD25 and Glut1 to attenuate IL-2 downstream signaling and glycolytic pathway, respectively. These changes subsequently modulate Th1, Th2, Th17, and Treg cell differentiation. It is somewhat counter-intuitive that a supply of exogenous glucosamine would actually exert a negative effect on N-glycosylation of glycoproteins such as CD25. Nonetheless, CD25 con-
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