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Authors

Li, Carey F
Zhou, Raymond W
Mkhikian, Haik
[et al.](#)

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Hypomorphic *MGAT5* polymorphisms promote multiple sclerosis cooperatively with *MGAT1* and interleukin-2 and 7 receptor variants

Carey F. Li^{a,c}, Raymond W. Zhou^b, Haik Mkhikian^b, Barbara L. Newton^a, Zhaoxia Yu^d, and Michael Demetriou^{a,b,c,*}

^aDepartment of Neurology, University of California, Irvine, CA 92869, USA

^bDepartment of Microbiology & Molecular Genetics, University of California, Irvine, CA 92869, USA

^cInstitute for Immunology, University of California, Irvine, CA 92869, USA

^dDepartment of Statistics, University of California, Irvine, CA 92869, USA

Abstract

Deficiency of the Golgi N-glycan branching enzyme *Mgat5* in mice promotes T cell hyperactivity, endocytosis of CTLA-4 and autoimmunity, including a spontaneous multiple sclerosis (MS)-like disease. Multiple genetic and environmental MS risk factors lower N-glycan branching in T cells. These include variants in interleukin-2 receptor- α (*IL2RA*), interleukin-7 receptor- α (*IL7RA*), and *MGAT1*, a Golgi branching enzyme upstream of *MGAT5*, as well as vitamin D3 deficiency and Golgi substrate metabolism. Here we describe linked intronic variants of *MGAT5* that are associated with reduced N-glycan branching, CTLA-4 surface expression and MS ($p = 5.79 \times 10^{-9}$, $n = 7,741$), the latter additive with the *MGAT1*, *IL2RA* and *IL7RA* MS risk variants ($p = 1.76 \times 10^{-9}$, OR = 0.67–1.83, $n = 3,518$).

Keywords

Multiple sclerosis; N-glycosylation; *MGAT5*; *MGAT1*; Interleukin-7 receptor; Interleukin-2 receptor

1. Introduction

Multiple sclerosis (MS) is a complex trait disease, whereby multiple genetic and environmental factors combine to determine MS susceptibility (Steinman, 2001; Hafler et al., 2007; Smolders et al., 2008). The majority of genetic variants that increase disease risk appear to be present in loci involved with regulating the immune response, such as the major histocompatibility complex locus (*HLA*) and interleukin receptors *IL2RA* and *IL7RA* (Gregory et al., 2007; Lundmark et al., 2007; Schmidt et al., 2007; Maier et al., 2009a,b;

*Corresponding author at: Department of Neurology, University of California, Irvine, CA 92869, USA. Tel.: + 1 949 824 9775; fax: + 1 949 824 9847. mdemetri@uci.edu (M. Demetriou).

International Multiple Sclerosis Genetics Consortium and Wellcome Trust Case Control Consortium, 2011). In addition to the genetic component, environmental factors such as exposure to sunlight are also significant determinants in MS risk (Smolders et al., 2008). The importance of the effects from the environment is most notably demonstrated in the case of monozygotic twins that are discordant for MS ~70% of the time despite virtually no differences in their genome, transcriptome and epigenetic profiles (Ebers et al., 1986; Baranzini et al., 2010). Evidence supporting the role of the environment in disease risk includes studies involving vitamin D3, where serum levels correlate with sunlight exposure, vary along the earth's longitudinal axis, and inversely correlate with MS susceptibility and severity (van der Mei et al., 2003; Soilu-Hanninen et al., 2005; Munger et al., 2006).

Understanding how genetic and environmental risk factors combine at the molecular level to promote loss of self-tolerance in MS is critical to identifying safe and efficacious therapies. In this regard, we recently reported that multiple genetic and environmental factors converge to disturb protein N-glycosylation of T cells in MS (Mkhikian et al., 2011). In mice, the Golgi N-glycan pathway is a critical modulator of T cell function and autoimmune disease (Grigorian et al., 2009). N-acetylglucosamine (GlcNAc) branched N-glycans attached to cell surface immuno-modulatory receptors are bound by a multimeric family of mammalian lectin proteins, called galectins (Dennis et al., 2009). This network of crosslinked galectins and glycoproteins form a molecular lattice on the cell surface that regulates surface concentration, lateral distribution, and signaling strength of the bound glycoproteins (Demetriou et al., 2001; Morgan et al., 2004; Chen et al., 2007; Grigorian et al., 2007; Lau et al., 2007; Lee et al., 2007; Grigorian and Demetriou, 2011). The Golgi enzymes *Mgat1*, 2, 4a/b, 5, along with metabolic production of their shared substrate UDP-GlcNAc, control the degree of N-glycan branching, the strength of the lattice and cell growth and differentiation (Grigorian et al., 2007; Lau et al., 2007; Grigorian et al., 2012a). For example, murine *Mgat5* deficiency results in hyperactive TCR clustering and signaling, surface loss of the anti-autoimmune receptor CTLA-4, pro-inflammatory T_H1 differentiation and development of spontaneous autoimmunity (Demetriou et al., 2001; Morgan et al., 2004; Lau et al., 2007; Grigorian et al., 2011). In the PL/J background, N-glycan branching deficiency promotes a spontaneous MS-like disease characterized by inflammatory demyelination and neurodegeneration (Lee et al., 2007). In MS, multiple genetic and environmental risk factors converge to disturb N-glycan branching by altering *MGATI* expression/activity (Mkhikian et al., 2011). These include genetic variants in *MGATI* ($V_A V_{T-T}$ haplotype: rs7726005, rs2070924, rs2070925), *IL2RA* (rs2104286) and *IL7RA* (rs6897932), as well as environmental factors consisting of vitamin D3 and metabolic production of UDP-GlcNAc. These interact with a polymorphism in *CTLA-4* (Thr17Ala, rs231775) that reduces the number of N-glycans attached to the protein by 50% to suppress surface expression of CTLA-4 in human T cell blasts (Anjos et al., 2002; Maurer et al., 2002). Supplementing cells or mice with the monosaccharide N-acetylglucosamine (GlcNAc) enhances UDP-GlcNAc production and N-glycan branching to reverse the T cell hyperactivity and/or autoimmunity induced by genetic deficiencies in N-glycan branching (Grigorian et al., 2007, 2011).

A recent genome-wide association study (GWAS) screening for variants that alter MS severity in Europeans identified two linked intronic *MGAT5* polymorphisms as their top hits (rs4953911 and rs3814022) (Brynedal et al., 2010). Although this association was not fully replicated in other cohorts for disease severity, whether rs4953911 and rs3814022 are involved in MS susceptibility remains unknown (Esposito et al., 2011; International Multiple Sclerosis Genetics Consortium, 2011). For example, the largest GWAS of MS risk done to date did not utilize a SNP chip that contained these two *MGAT5* SNPs (International Multiple Sclerosis Genetics Consortium and Wellcome Trust Case Control Consortium, 2011). In addition to disease risk, the ability of these SNPs to alter N-glycan branching and thus cell surface concentration of immuno-modulatory receptors have yet to be investigated. Here, we report that *MGAT5* rs4953911 and rs3814022 are associated with reduced N-glycan branching and CTLA-4 surface expression as a consequence of decreased *MGAT5* expression in blasting T cells, a phenotype rescued by metabolic supplementation of cells with GlcNAc. Moreover, we find that *MGAT5* rs4953911 and rs3814022 associate with MS in two independent MS case-control cohorts. Risk of MS was more strongly associated with *MGAT5* rs4953911 and as expected, combining this stronger variant with other known variants that also lower N-glycan branching, namely *MGATI* V_AV_{T-T}, *IL2RA* (rs2104286) and *IL7RA* (rs6897932), additively increased the risk of MS (Gregory et al., 2007; Lundmark et al., 2007; Maier et al., 2009a,b; Mkhikian et al., 2011). Our results provide additional evidence that defective N-glycosylation plays a critical role in MS and further support the potential of metabolic supplementation to the N-glycan pathway with GlcNAc as a therapeutic tool for MS treatment and risk management.

2. Materials and methods

2.1. Human subjects

All human subjects used for both T cell experiments and genetic correlation studies are Caucasian (non-Hispanic) and include both males and females. For T cell experiments, human whole blood was collected from healthy control individuals through the Research Blood Donor Program serviced by the Institute for Clinical & Translational Science (ICTS) at University of California, Irvine (UCI). For genetic correlation studies, we examined two independent Caucasian MS case-control cohorts, one from North America and one from Europe. Cohort 1 cases (n = 1201 total) consisted of unrelated, non-Hispanic Caucasians with clinically definite MS obtained at UCI (n = 82) or from across the United States by the Multiple Autoimmune Disease Genetics Consortium (n = 176, MADGC, www.madgc.org) and the Accelerated Cure Project for Multiple Sclerosis (n = 943, www.acceleratedcure.org). Cohort 1 controls (n = 2377 total) were unrelated, non-diseased, non-Hispanic Caucasians obtained at UCI (n = 119), which include individuals used for T cell experiments, and from across North America by North American Rheumatoid Arthritis Consortium (n = 192, NARAC, www.naracdata.org), the Type1 Diabetes Genetics Consortium (n = 1199, T1DGC, www.t1dgc.com), the Genetics of Kidneys in Diabetes (GoKinD) (n = 746, www.niddkrepository.org), and publically available datasets (n = 121) from the HapMap Project (www.hapmap.org) and 1000 Genomes Project (www.1000genomes.org). T1DGC and GoKinD DNA samples were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Cohort 2 data was largely obtained from

publically available datasets. Cases (n = 1913 total) were European [Sweden (n = 988), France (n = 568) and Italy (n = 357)] MS subjects previously genotyped for *MGAT5* rs4953911 and rs3814022 (Brynedal et al., 2010). Cohort 2 controls (n = 2338 total) were Europeans from the 'British 1958 Birth Cohort' (n = 1480) and T1DGC (n = 858). Both control cohorts were in Hardy Weinberg Equilibrium (HWE). The sample size for individual alleles reported in Tables 1 and 2 varies marginally from these numbers due to failed genotyping for that specific allele in a few cases and/or controls.

2.2. T cell isolation and analysis

Human T cells from healthy control individuals (n = 11 total, 4 females, 7 males; ages 22–55, mean age = 33) were isolated from whole blood using the RosetteSep Human CD4⁺ T cell enrichment kit (StemCell Technologies) and stimulated with PMA (1 ng/ml) and ionomycin (125 ng/ml) for 5 days in culture. In GlcNAc supplementation experiments, 40 mM GlcNAc (Ultimate Glucosamine, Wellesley Therapeutics Inc.) and 10 mM uridine (Sigma) were added to cells in culture at Day 0. For flow cytometry, after normalizing for cell number, CD4⁺ T cells were stained with 2 ug/ml L-PHA-FITC (Vector Labs) to detect β 1,6-GlcNAc branched N-glycans. T cells were also co-stained with CD4-PE-Cy5 and CD25-PE (eBioscience) to mark CD4⁺ T cell blasts. For CTLA-4 cell surface measurements, T cells were stained with anti-CTLA-4-PE or its respective PE-labeled isotype control (eBioscience), and final CTLA-4 values were adjusted for nonspecific binding by isotype subtraction. Fluorescence levels were measured by flow cytometry using BD's FACSCalibur flow cytometer. Post-acquisition analysis was performed using FlowJo software. For RNA analysis, CD4⁺ T cells were stimulated as above and harvested at the indicated time points. RNA was isolated using RNeasy Mini Kit (Qiagen) and used to generate cDNA using M-MLV Reverse Transcriptase (Invitrogen). Gene expression analysis was performed using real-time qPCR with TaqMan Gene Expression Assays (ABI) specific for human *MGAT5* (Hs00327733_s1) and beta-actin (Hs99999903_m1). The Ct method was used to calculate relative fold enrichment in *MGAT5* RNA expression using beta-actin for normalization.

2.3. SNP analysis

Single nucleotide polymorphism (SNP) genotyping was performed using TaqMan SNP Genotyping Assays (ABI) as previously described (Mkhikian et al., 2011). TaqMan SNP genotyping assays utilized for *MGAT5* SNPs rs4953911 and rs3814022 are C__3258342_10 and C__2163491_10, respectively. Genotyping assays for *IL2RA* (rs2104286) and *IL7RA* (rs6897932) are C__16095542_10 and C__2025977_10, respectively. The *MGAT1* IV_AV_{T-T} haplotype (rs7726005, rs2070924 and rs2070925) was genotyped using two custom TaqMan primer/probe sets targeting rs7726005 and rs2070924 as previously described (Mkhikian et al., 2011). The two *MGAT1* V_{T-T} SNPs (rs2070924 and rs2070925) are 10nt apart and sequenom genotyping of 1387 subjects revealed 100% concordance. The genotyping success rate for all 7 SNPs examined was > 99% in all TaqMan genotyped cohorts except for *MGAT5* rs4953911 in MS cohort 1 (> 98%). Concordance for duplicate genotyping was determined by re-genotyping all 6 SNPs and revealed 100% concordance (n = 735) for all SNPs except *MGAT5* rs4953911 and rs3814022 (99.5%, n = 374 and n = 376, respectively).

2.4. Statistical analysis

Individual p-values for association studies in Table 1 for North American cohort 1 and European cohort 2 are one-sided and were computed using the Cochran–Armitage test, while combined p-values were generated using Fishers method. For genetic interaction studies in Table 2 for cohort 1, *MGAT5**T (rs4953911), *IL2RA**T (rs2104286) and *IL7RA**C (rs6897932) were coded as 0, 1 or 2 for the number of risk alleles. In addition, *MGATI* IV_AV_{T-T} (rs7726005, rs2070924 and rs2070925) non-carriers were coded as 0, while carriers with less than four total risk alleles for *IL2RA**T + *IL7RA**C were coded as 1 and carriers with exactly four risk alleles for *IL2RA**T + *IL7RA**C as -1. The one-sided p-value for this trend analysis was computed using the Cochran–Armitage test. Multiple logistic regression analysis was performed using the following four terms: *MGAT5**T, *MGATI* IV_AV_{T-T}, *IL2RA**T + *IL7RA**C and the interaction between *MGATI* IV_AV_{T-T} and *IL2RA**T + *IL7RA**C. Alleles were coded as 0, 1 or 2 for the number of risk alleles, except for *MGATI* IV_AV_{T-T} which was coded as 0 or 1 for carrier status. From this fitting, a likelihood ratio test with four degrees of freedom was used to generate a multiple logistic regression p-value. Odds ratios for carriers with varying numbers of risk alleles were determined from the logistic regression coefficient.

3. Results

3.1. Downregulation of N-glycan branching and surface CTLA-4 by *MGAT5* genetic variants

MGAT5 rs4953911 and rs3814022 are in strong linkage disequilibrium (Lewontin's $D' = 0.98$ and Pearson's correlation coefficient $r = 0.90$), and both reside within intron 2 of the *MGAT5* gene. Direct assessment of the biological effect of intronic SNPs is difficult; therefore we correlated changes in *MGAT5*-induced β 1,6- GlcNAc branching in CD4⁺ T cells isolated from healthy donors containing the *MGAT5* risk variants or their common allele counterparts. For the sake of simplicity and to provide the clearest comparison, we analyzed cells from individuals who were homozygous for both rs4953911 and rs3814022, or homozygous for their common alleles. In addition, to minimize variability, we also matched cells for the other MS variants known to alter N-glycan branching, namely *MGATI* V_AV_{T-T}, *IL2RA* (rs2104286) and *IL7RA* (rs6897932) (Mkhikian et al., 2011). Cell surface β 1,6-GlcNAc branching was assessed by flow cytometry with L-PHA (*Phaseolus vulgaris*, leucoagglutinin), a plant lectin that binds specifically to β 1,6-GlcNAc-branched N-glycans and serves as a quantitative marker of N-glycan branching (Cummings and Kornfeld, 1982; Demetriou et al., 2001). In non-activated, resting T cells, very little difference in L-PHA binding was seen on average between rs4953911/rs3814022 (TT/GG) homozygotes and the common allele homozygotes (AA/CC) (Fig. 1A). In contrast, activated T cell blasts homozygous for rs4953911/rs3814022 displayed significant decreases in L-PHA binding over the homozygous controls (~21% average decrease, $p = 0.0002$) (Fig. 1B). Consistent with this decrease in N-glycan branching, *MGAT5* RNA levels are also lower in activated T cells homozygous for the *MGAT5* variants when compared to the common alleles, with the differences peaking at nine hours post stimulation (~35% average decrease) (Fig. 1C). This suggests that rs4953911/rs3814022 SNPs have a role in modulating N-glycan branching in T cells in the activated state, and this modulation likely occurs via effects on *MGAT5* RNA expression levels.

We then investigated more closely the changes in N-glycosylation on a day-by-day basis for the *MGAT5* variants. Alterations in L-PHA binding associated with rs4953911/rs3814022 did not peak until Days 4–5 following stimulation (~20–27% average decrease) (Fig. 1D). This is the period of the T cell growth cycle when up-regulation of anti-autoimmune CTLA-4 at the cell surface is required to induce growth arrest, a phenotype regulated by N-glycan branching (Grigorian et al., 2007; Lau et al., 2007). Thus, we investigated the possibility of these *MGAT5* variants altering CTLA-4 surface levels in individuals matched for the CTLA-4 Thr17Ala allele (rs231775), which is known to alter the number of N-glycans attached to CTLA-4 (Anjos et al., 2002). Indeed, rs4953911/rs3814022 homozygosity (TT/GG) was also associated with a reduction in CTLA-4 surface expression in 5-day old T cell blasts compared to average values for its common allele control counterparts (AA/CC) (~58% average decrease, $p = 0.014$) (Fig. 2A). Supplementing T cells homozygous for the *MGAT5* variants with GlcNAc restored L-PHA binding (Fig. 2B) and CTLA-4 surface expression (Fig. 2A) to that of control homozygotes, confirming that the observed differences resulted from alterations in branching. These results are consistent with the *MGAT5* SNPs playing a role in MS severity and in line with published reports which demonstrated murine *Mgat5* deficiency lowers CTLA-4 surface expression and promotes MS-like disease in mice (Demetriou et al., 2001; Lau et al., 2007; Brynedal et al., 2010).

3.2. Association of *MGAT5* variants with MS risk

MGAT5 rs4953911 and rs3814022 were first identified in a case-only MS study ($n = 1040$) screening for polymorphisms associated with disease severity, with reported p -values of 1.5×10^{-7} and 2.8×10^{-6} , respectively (Brynedal et al., 2010). However, our previous work demonstrating a link between decreases in N-glycan branching and risk of MS in humans and MS-like disease in mice suggests that *MGAT5* rs4953911 and rs3814022 should also increase the risk of MS (Demetriou et al., 2001; Lee et al., 2007; Mkhikian et al., 2011). To investigate this hypothesis, we genotyped both polymorphisms in a non-Hispanic Caucasian cohort of 1201 MS cases and 2377 controls from North America (cohort 1). *MGAT5* rs4953911 and rs3814022 both associated with MS, with p -values of 7.77×10^{-7} and 6.50×10^{-6} , respectively (Table 1). The two alleles are in high LD and haplotype analysis gave $p = 4.52 \times 10^{-6}$ for the rare haplotype, providing no additional information over the point analysis. Association of the two variants was replicated in a second independent cohort consisting of European MS cases ($n = 1913$) previously genotyped for both *MGAT5* SNPs (Brynedal et al., 2010) and separate European controls from the '1958 British Birth Cohort' and the Type-1 Diabetes Genetics consortium ($n = 2338$). P -values of 3.23×10^{-4} and 4.21×10^{-4} for rs4953911 and rs3814022 were observed in this cohort, respectively. Combining p -values from the two cohorts produced genome-wide significance for rs4953911 ($p = 5.79 \times 10^{-9}$) and near genome-wide significance for rs3814022 ($p = 5.67 \times 10^{-8}$).

3.3. Additive genetic risk of *MGAT5* SNPs with *MGAT1*, *IL2RA* and *IL7RA* variants in MS

Next we examined whether combining the stronger associated MS risk allele rs4953911 with other known variants that also lower N-glycan branching and promote MS increases disease risk beyond what is seen with the individual variants. The *MGAT1* V_AV_{T-T} haplotype (rs7726005, rs2070924, rs2070925), *IL2RA**T (rs2104286) and *IL7RA**G (rs6897932) all lower N-glycan branching and show point association with MS (Mkhikian et

al., 2011). However, *IL2RA**T and *IL7RA**C reduce N-glycan branching by downregulating *MGATI* mRNA; whereas the *MGATI* IV_AV_{T-T} haplotype enhances *MGATI* mRNA expression yet lowers N-glycan branching by sequestering the shared substrate UDP-GlcNAc from *MGAT5*. These opposing effects on *MGATI* mRNA cancel each other out and result in enhanced N-glycan branching and reduced risk of MS when *MGATI* V_AV_{T-T}, *IL2RA**T and *IL7RA**G are combined. To account for this interaction in a trend analysis, *IL2RA**T and *IL7RA**C were both coded as 0, 1 or 2 for the number of risk alleles, while *MGATI* IV_AV_{T-T} non-carriers were coded 0, carriers with *IL2RA**T + *IL7RA**C < 4 as 1, and carriers with *IL2RA**T + *IL7RA**C = 4 as -1. *MGAT5**T (rs4953911) was also encoded as 0, 1 and 2, as this variant is expected to reduce N-glycan branching irrespective of whether *MGATI* is increased or decreased. A trend analysis of our North American MS cohort (cohort 1) with this coding gives $p = 2.78 \times 10^{-9}$ (one-sided Cochran–Armitage test, Table 2). The odds ratio (OR) increases from 0.67 for individuals with 1 variant to 1.83 for those with 6 variants. Similarly, using the logistic regression coefficient to estimate the OR predicts an OR = 1.22 for each additional variant added.

We also fit a multiple logistic regression with four terms: *MGAT5**T (rs4953911), *MGATI* IV_AV_{T-T}, *IL2RA**T + *IL7RA**C and the interaction between *MGATI* IV_AV_{T-T} and *IL2RA**T + *IL7RA**C. In this analysis, *MGATI* IV_AV_{T-T} was coded 0 or 1 for carrier status, while the others were coded as 0, 1 or 2 for the number of risk alleles. For cohort 1, the p-value based on the likelihood ratio test with four degrees of freedom is 1.76×10^{-9} . Importantly, the p-value of the three terms without *MGAT5**T (rs4953911) is 1.87×10^{-5} and comparing the two models indicates that *MGAT5**T (rs4953911) is still significant ($p = 2.59 \times 10^{-6}$) after taking the other three terms into consideration. Thus, *MGAT5**T (rs4953911) provides additional information for MS risk. For cohort 2, data for *MGATI* IV_AV_{T-T}, *IL2RA**T and *IL7RA**C are not publicly available for the MS cases and therefore could not be analyzed with *MGAT5**T (rs4953911) for combined effects. However, the results in cohort 1 are consistent with additive effects of *MGAT5* rs4953911 and the other known MS risk alleles that also lower N-glycan branching.

4. Discussion

Elucidating the many factors which drive autoimmune diseases such as MS remains a challenging prospect due to the complexities of various interacting pathways. In the case of MS, the crosstalk between genetics and the environment dictates the need for multiple factors to be considered at once in order to correctly assess their true impact on disease risk and activity (Mkhikian et al., 2011; Grigorian et al., 2012b). In this report, we have shown that the *MGAT5* intronic variants rs4953911 and rs3814022 correlate with lower N-glycan branching, reduced surface CTLA-4 in human CD4⁺ T cell blasts, and associate with MS. In addition, the stronger MS risk allele rs4953911 additively combines with other known MS risk alleles that also lower N-glycan branching (i.e. *MGATI* V_AV_{T-T}, *IL2RA*, and *IL7RA*) to further enhance disease risk. Consistent with association in MS, we have recently reported family based association of *MGAT5* rs4953911 and rs3814022 with Type 1 diabetes (5.8×10^{-3} and $p = 1.5 \times 10^{-3}$, respectively) via analysis of 2395 Caucasian families from the Type 1 Diabetes Genetics Consortium (Yu et al., in press). *MGAT5* RNA levels are lower in rs4953911/rs3814022 homozygotes, suggesting these SNPs may cause a change in splicing

efficiency and/or transcriptional regulation leading to a defect in *MGAT5* expression and thus decreased N-glycan branching. However, strong linkage disequilibrium with another polymorphism that serves as the primary functional variant cannot be excluded.

The identification and characterization of *MGAT5* (rs4953911/rs3814022) bring the number of independent MS genetic and environmental risk factors demonstrated to dysregulate N-glycosylation and CTLA-4 surface expression in T cell blasts to seven. These are *MGAT5* (rs4953911, rs3814022), *MGAT1* IV_AV_{T-T} (rs7726005, rs2070924, rs2070925), *IL2RA* (rs2104286), *IL7RA* (rs6897932), *CTLA-4* (rs231775), vitamin D3 deficiency and metabolic production of UDP-GlcNAc (Mkhikian et al., 2011). The largest GWAS of MS risk to date recently increased the number of MS associated loci to > 50 (International Multiple Sclerosis Genetics Consortium and Wellcome Trust Case Control Consortium, 2011). Based on the biology of the nearest gene, many of these are likely to also dysregulate N-glycosylation. For example, two loci are in/near vitamin D3 metabolic enzymes (*CYP27B1*, *CYP24A1*), while another is in/near the *IL-7* cytokine gene. Moreover, many genomic regions were near genes that control TCR signal strength, a critical determinant of N-glycan branching and thus CTLA-4 surface retention in T cell blasts (Demetriou et al., 2001; Lau et al., 2007; Chen et al., 2009). These include *CD37*, *CD40*, *CD58*, *CD80*, *CD86*, *CLECLI*, *CBLB*, *MALT1*, *TAGAP* and *MAPK1*. The other major category of loci identified contained genes for cytokines and their signaling machinery, including *IL12A*, *IL12B*, *IL12RB1*, *IL22RA2*, *IRF8*, *TNFRSF1A*, *TNFRSF14*, *TNFSF14*, *TYK2* and *STAT3*. We are currently examining these pathways and it appears that a number of these may also regulate N-glycan branching. With further analysis, a significant proportion of the polymorphisms associated with MS may directly or indirectly regulate N-glycosylation to affect T cell growth and differentiation. Such a result is supported by our data in mice, where *Mgat5* and N-glycan branching deficiency induce a spontaneous MS-like disease (Demetriou et al., 2001; Lee et al., 2007). To our knowledge, this has not been demonstrated for any other MS-associated gene outside of HLA-TCR double transgenic mice (Madsen et al., 1999). Finally, continued expansion of the number of MS associated loci known to dysregulate N-glycosylation will further validate the potential use of GlcNAc as a therapeutic tool for the treatment and risk management of MS.

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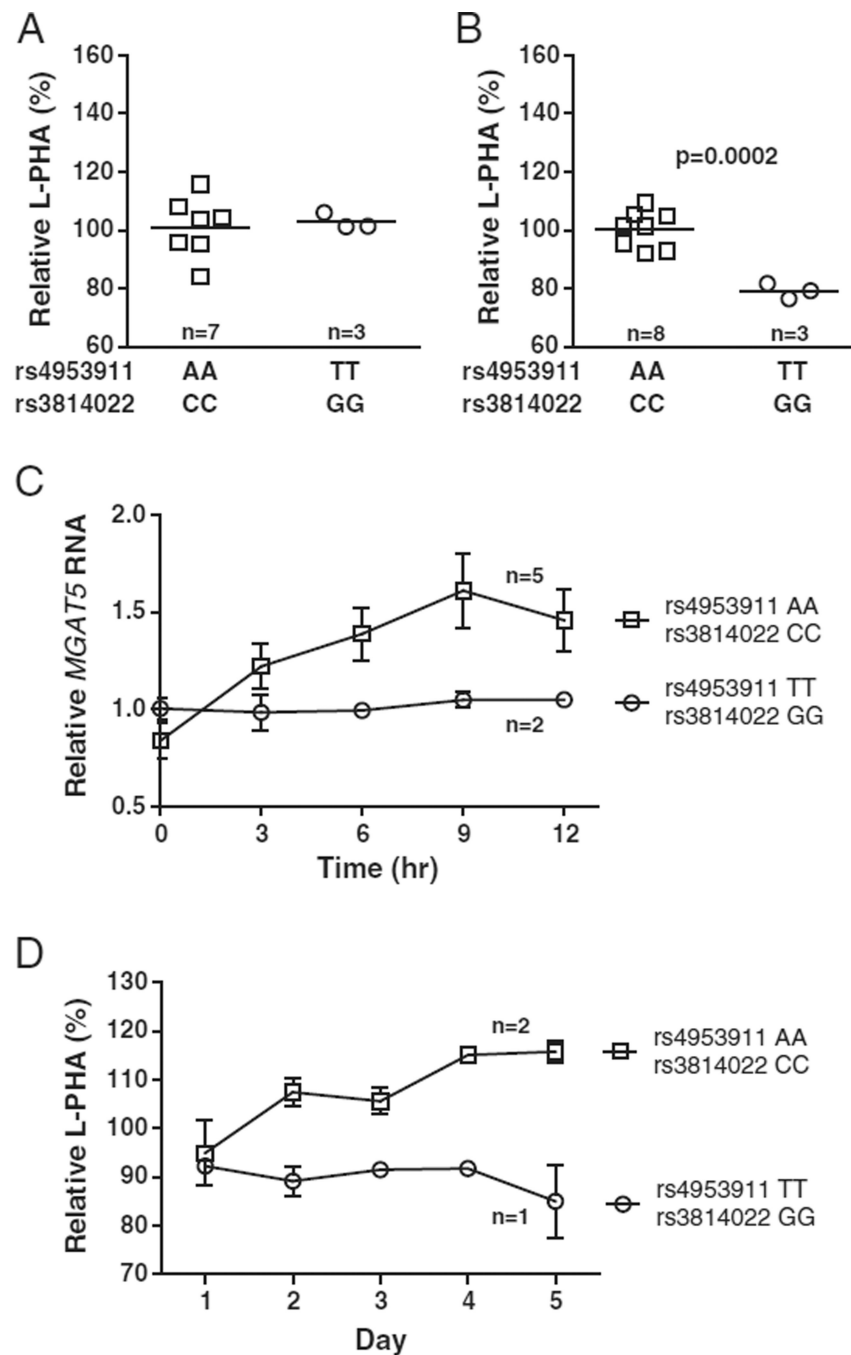


Fig. 1. *MGAT5* rs4953911/rs3814022 reduce GlcNAc branching and *MGAT5* RNA in activated T cells. (A) Scatterplot of L-PHA binding by flow cytometry in non-activated, resting human T cells. Purified CD4⁺ T cells homozygous for rs4953911/rs3814022 (TT/GG) or their common allele control counterparts (AA/CC) were rested 5 days in culture before analysis for surface levels of GlcNAc branching. Each data point (square or circle) represents the average of triplicate L-PHA measurements for each individual. Relative L-PHA values were generated by normalization to the average of the control group (set at 100%). The horizontal

line across each data group represents the average L-PHA value of that entire group. (B) Scatterplot of L-PHA binding in activated CD4⁺ CD25⁺ T cell blasts. Purified CD4⁺ T cells were activated with PMA (1 ng/ml) and ionomycin (125 ng/ml) for 5 days in culture. L-PHA binding measurements and analysis were performed as in (A). (C) Time course analysis of *MGAT5* RNA levels in activated CD4⁺ T cells. CD4⁺ T cells were stimulated as in (B) for 0, 3, 6, 9, and 12 h before harvesting for analysis. Each line represents combined average *MGAT5* RNA values for the specified group. Relative *MGAT5* RNA values were generated by normalization to the average value of rs4953911/rs3814022 homozygotes within each hour (set at 1). (D) Time course analysis of L-PHA binding changes in CD4⁺ CD25⁺ T cell blasts. Activated cells were stimulated as in (B) and harvested each day for a total of 5 days. L-PHA binding measurements and analysis were performed as in (A). Each line represents combined average branching values for the specified group. Relative L-PHA values were generated by normalization to a control individual at Day 1 (set at 100%). For all panels, n is the total number of unique individuals analyzed per group. Error bars represent stand error of the mean (SEM). P-values were generated using one-tailed unpaired t-tests.

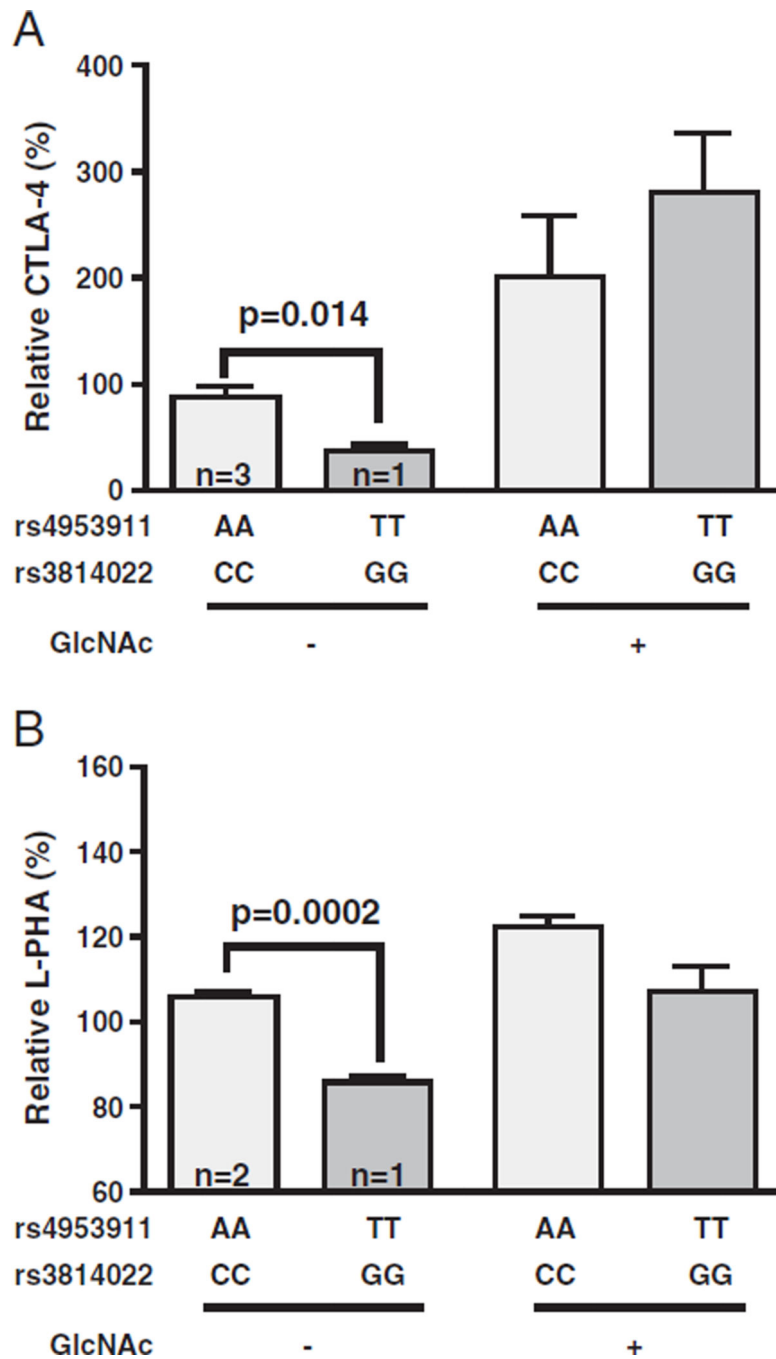


Fig. 2. *MGAT5* rs4953911/rs3814022 downregulate CTLA-4 surface expression. Bar graphs of surface CTLA-4 positive cells (A) or L-PHA binding levels (B) in individuals homozygous for the common *MGAT5* alleles (AA and CC for rs4953911 and rs3814022, respectively) or the average values from one individual homozygous for both variant alleles (TT and GG, respectively) are shown. CD4⁺ T cells were activated as in Fig. 1B, with or without treatment with 40 mM GlcNAc and 10 mM uridine. Cell surface expression of CTLA-4 or L-PHA binding on CD4⁺ T cell blasts were measured by flow cytometry. Relative L-PHA

values and relative percentage of cells positive for CTLA-4 surface expression were calculated by normalization to control individuals (set at 100%). In addition, CTLA-4 values were adjusted by subtracting for their respective isotype control. $n = 3$ and $n = 2$ for the number of unique control individuals in panels (A) and (B), respectively. Error bars represent standard error of the mean (SEM). P-values were generated using one-tailed unpaired t-tests of triplicate staining.

Table 1

MGAT5 variants in multiple sclerosis.

		Variant frequency		p-value	OR [95% CI]
	Control (n)	MS (n)			
<i>MGAT5</i> *T (rs4953911)	Cohort 1	0.302 (2364)	0.357 (1183)	7.77×10^{-7}	1.31 [1.17–1.47]
	Cohort 2	0.288 (2338)	0.323 (1856)	3.23×10^{-4}	1.17 [1.07–1.29]
			Combined p-value:	5.79×10^{-9}	
<i>MGAT5</i> *G (rs3814022)	Cohort 1	0.263 (2363)	0.309 (1201)	6.50×10^{-6}	1.29 [1.15–1.45]
	Cohort 2	0.254 (2331)	0.286 (1897)	4.21×10^{-4}	1.18 [1.07–1.29]
			Combined p-value:	5.67×10^{-8}	

Reported are one-sided p-values computed using the Cochran–Armitage test of North American (cohort 1) and European (cohort) non-Hispanic Caucasian case-control cohorts. All individual control cohorts are in HWE. P-values were combined using Fishers method. OR=odds ratio. 95% CI = 95% confidence intervals.

Table 2

MGAT5 with *IL2RA*, *IL7RA* and *MGAT1* *IVAVT-T* in multiple sclerosis.

Variables:	0	1	2	3	4	5	6
Controls	2	62	281	727	817	387	68
Cases	0	21	95	328	427	242	61
OR (x vs rest) ^c	-	0.67	0.64	0.86	1.07	1.31	1.83
P-value ^d							2.78×10^{-9}
OR (x + 1 vs x) ^d							1.22 [1.14–1.30]
Multiple logistic regression p-value ^b							1.76×10^{-9}

Analysis of Cohort 1 (North American non-Hispanic Caucasian case-control MS cohort).

^a One-sided p-value computed using the Cochran–Armitage test for trend with *MGAT5**T (rs4953911), *IL2RA**T (rs2104286) and *IL7RA**C (rs6897932) coded as 0, 1 or 2 and *MGAT1* *IVAVT-T* (rs7726005, rs2070924 and rs2070925) non-carriers coded as 0, carriers with *IL2RA**T + *IL7RA**Cb4 as 1 and carriers with *IL2RA**T + *IL7RA**C = 4 as –1.

^b p-value from the likelihood ratio test (4 degrees of freedom) from fitting a multiple logistic regression with 4 terms: *MGAT5**T, *MGAT1* *IVAVT-T*, *IL2RA**T + *IL7RA**C and the interaction between *MGAT1* *IVAVT-T* and *IL2RA**T + *IL7RA**C. Variables were coded as 0, 1 or 2 for the number of risk alleles, except *MGAT1* *IVAVT-T* which was coded as 0 or 1 for carrier status.

^c Odds ratio (OR) for carriers with X risk alleles versus carriers with other numbers of risk alleles.

^d OR for X + 1 versus X determined from the logistic regression coefficient.