

N-Acetylglucosaminyltransferase V (Mgat5)-Mediated *N*-Glycosylation Negatively Regulates Th1 Cytokine Production by T Cells¹

Rodney Morgan,* Guoyan Gao,[‡] Judy Pawling,[†] James W. Dennis,[†] Michael Demetriou,[‡] and Baiyong Li^{2*}

The differentiation of naive CD4⁺ T cells into either proinflammatory Th1 or proallergic Th2 cells strongly influences autoimmunity, allergy, and tumor immune surveillance. We previously demonstrated that β 1,6GlcNAc-branched complex-type (*N*-acetylglucosaminyltransferase V (Mgat5)) *N*-glycans on TCR are bound to galectins, an interaction that reduces TCR signaling by opposing agonist-induced TCR clustering at the immune synapse. Mgat5^{-/-} mice display late-onset spontaneous autoimmune disease and enhanced resistance to tumor progression and metastasis. In this study we examined the role of β 1,6GlcNAc *N*-glycan expression in Th1/Th2 cytokine production and differentiation. β 1,6GlcNAc *N*-glycan expression is enhanced by TCR stimulation independent of cell division and declines at the end of the stimulation cycle. Anti-CD3-activated splenocytes and naive T cells from Mgat5^{-/-} mice produce more IFN- γ and less IL-4 compared with wild-type cells, the latter resulting in the loss of IL-4-dependent down-regulation of IL-4R α . Swainsonine, an inhibitor of Golgi α -mannosidase II, blocked β 1,6GlcNAc *N*-glycan expression and caused a similar increase in IFN- γ production by T cells from humans and mice, but no additional enhancement in Mgat5^{-/-} T cells. Mgat5 deficiency did not alter IFN- γ /IL-4 production by polarized Th1 cells, but caused an ~10-fold increase in IFN- γ production by polarized Th2 cells. These data indicate that negative regulation of TCR signaling by β 1,6GlcNAc *N*-glycans promotes development of Th2 over Th1 responses, enhances polarization of Th2 cells, and suggests a mechanism for the increased autoimmune disease susceptibility observed in Mgat5^{-/-} mice. *The Journal of Immunology*, 2004, 173: 7200–7208.

T cell activation and differentiation are accompanied by changes in the expression of glycosyltransferases and glycosidases, the Golgi enzymes that remodel *N*- and *O*-linked glycans (1). Conversion of activated T cells to memory cells or differentiation of Th cells into Th1 and Th2 subsets change cell surface glycosylation profiles and thereby influence trafficking (2–5). For example, differential expression of sialyl and sulfo-LeX sequences found at the termini of poly-*N*-acetyl-lactosamine repeats on cell surface *O*-glycans bind to L-, E-, and P-selectins and regulate lymphocyte trafficking to lymphoid organs and sites of inflammation, including that of memory Th cells (6).

The expression of the branching β 1,6-*N*-acetylglucosaminyltransferase V (Mgat5)³ *N*-linked glycans increases after T cell activation (7). Mgat5 is a medial Golgi enzyme that transfers GlcNAc from UDP-GlcNAc to the OH on carbon 6 of the α 1,6-linked mannose of *N*-glycans present on newly synthesized glycoproteins. Glycoproteins are then transported through the trans-Golgi where

the *N*-glycans are extended with *N*-acetyl-lactosamine and poly-*N*-acetyl-lactosamine and capped with sialic acid, fucose, and sulfate. Mgat5 branched *N*-glycans are the preferred intermediates for extension with poly-*N*-acetyl-lactosamine and serve as ligands for the galectin family of *N*-acetyl-lactosamine-binding lectins (8, 9). The TCR subunits are *N*-glycosylated, a fraction of which are modified by Mgat5 and poly-*N*-acetyl-lactosamine and bind to galectin-3 (7, 10). The nonlectin N-terminal domain of galectin-3 mediates rapid conversion into pentamers in the presence of multivalent ligands, thereby cross-linking glycoproteins in proportion to ligand concentrations (11). Galectin cross-linking of Mgat5-modified cell surface glycoproteins forms a dynamic lattice that opposes agonist-dependent TCR clustering (7).

Mgat5^{-/-} embryos develop normally, but mutant mice display abnormal responses to extrinsic stimuli (7, 12), including T cell hypersensitivity in vitro and in vivo. Agonist-induced TCR clustering was enhanced in Mgat5^{-/-} T cells, which lowered the threshold for TCR-dependent tyrosine phosphorylation, actin microfilament reorganization, Ca²⁺ mobilization, and T cell proliferation. In vivo, Mgat5^{-/-} mice display enhanced delayed-type hypersensitivity, increased susceptibility to experimental autoimmune encephalomyelitis, an ~30% incidence of spontaneous autoimmune kidney glomerulonephritis between 12 and 18 mo of age (7), and delayed tumor progression in polyomavirus middle T transgenic mice (12); the latter may be due in part to enhanced antitumor immune activity. Susceptibility to autoimmune disease and antitumor immunity are strongly influenced by the relative differentiation of naive CD4⁺ T cells into either proinflammatory Th1 cells that secrete IFN- γ and TNF- β or anti-inflammatory Th2 cells that secrete IL-4, IL-5, IL-10, and IL-13 (13, 14). Enhancement of TCR signaling by increasing Ag dose or affinity promotes Th1 and inhibits Th2 differentiation (15–18). This suggests that the

*Department of Antibacterials, Immunology, and Cancer, Pfizer Global Research and Development, Groton, CT 06340; [†]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada; and [‡]Departments of Neurology and Microbiology and Molecular Genetics, University of California, Irvine, CA 92697

Received for publication March 23, 2004. Accepted for publication October 5, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported in part by grants from the National Institute of Allergy and Infectious Disease, National Institutes of Health (to M.D.), and the Canadian Institutes for Health Research (to J.W.D.).

² Address correspondence and reprint requests to Dr. Baiyong Li, Department of Antibacterials, Immunology, and Cancer, Pfizer Global Research and Development, Eastern Point Road, Groton, CT 06340. E-mail address: baiyong_li@groton.pfizer.com

³ Abbreviations used in this paper: Mgat5, *N*-acetylglucosaminyltransferase V; L-PHA, plant lectin leucoagglutinin.

lowered T cell activation thresholds produced by Mgat5 glycan deficiency may promote autoimmunity and antitumor immunity by directly increasing Ag-induced Th1 differentiation. To explore this possibility, we have examined the role of Mgat5 modified *N*-glycans in Th1/Th2 cell development. We demonstrate that expression of β 1,6GlcNAc-branched *N*-glycans selectively inhibits Th1 cell differentiation and enhances the polarization of Th2 cells.

Materials and Methods

Mice and cells

Mgat5^{-/-} mice on the 129 background were described previously (12). DO11.10 TCR transgenic mice in BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME). Murine CD4 T cells were isolated from spleens using the DETACHaBEAD Mouse CD4 Isolation Kit (DynaL Biotech, Great Neck, NY) according to the manufacturer's protocol. CD3⁺ T cells were isolated using a negative selection column (R&D Systems, Minneapolis, MN). To generate murine Th1 and Th2 cells, naive T cells were isolated from CD4 T cells using the CD62L MACS positive selection system (Miltenyi Biotec, Auburn, CA) and stimulated with plate-bound anti-CD3 (10 μ g/ml), soluble anti-CD28 (1 μ g/ml), IL-12, and anti-IL4 for Th1 cells or with anti-CD3 (10 μ g/ml), anti-CD28 (1 μ g/ml), IL-4, and anti-IFN- γ for Th2 cells. The cytokine concentrations and Abs used were described previously (19). For restimulation, cells were washed and stimulated with 1 μ g/ml anti-CD3. For plant lectin leucoagglutinin (L-PHA) staining of *in vitro* differentiated Th1 and Th2 cells, splenocytes from DO11.10/Rag1 knockout mice were stimulated with 300 ng/ml OVA peptide and differentiating cytokines as described above. Human naive T cells were isolated from peripheral blood from normal volunteers using an Accuspin PBMC isolation kit (Sigma-Aldrich, St. Louis, MO), then using a CD4⁺CD45RO⁻ subset kit (R&D Systems).

Cytokine measurement using Bioplex

Cells are routinely stimulated at 1 million/ml, and supernatant was collected at various times after stimulation. In some cases, splenocytes were stimulated with 1 μ g/ml anti-CD28 in combination with various concentrations of anti-CD3; in other cases, 1 μ g/ml anti-CD3 was immobilized to the plate, then combined with 2 μ g/ml anti-CD28 as stimulus. For restimulation, cells were washed and counted, and the same number of cells was plated per well. Cytokines were measured using Bioplex system (Bio-Rad, Hercules, CA); cytokine standard and reagent for measuring TNF- α were purchased from Upstate Biotechnology (Lake Placid, NY). For each experiment, triplicate samples were measured, and averages were taken for data analysis. In some cases, diluted samples were measured to fit the dynamic range of the standards, and cytokine concentrations in supernatant were calculated accordingly.

L-PHA and CFSE staining

Murine splenocytes or human T cells were stimulated as indicated in the figure legends and in some instances were treated with various concentrations of swainsonine and stained with FITC-conjugated L-PHA (Vector Laboratories, Burlingame, CA) for 15 min on ice, washed, and analyzed using FACS. A titration of L-PHA-FITC from 0.2 ng/ml to 200 μ g/ml was performed initially, and all subsequent staining was performed using 2 μ g/ml L-PHA-FITC. For CFSE staining and proliferation, purified CD3⁺ T cells were incubated with 5 μ M CFSE for 8 min at room temperature, washed, and stimulated with plate-bound anti-CD3 with or without anti-CD28. After 3 days of culture, the cells were washed, stained with L-PHA-PE and anti-CD4-PE-Cy5, and analyzed by FACS.

Cytokine receptor FACS

Purified CD3⁺ T cells from Mgat5^{+/+} and Mgat5^{-/-} were stimulated with 1 μ g/ml plate-bound wild-type anti-CD3 (clone 2C11) 48 h in the presence or the absence of rIL-4 (10 ng/ml), IL-12 (10 ng/ml), IFN- γ (10 ng/ml), anti-IL-4 (10 μ g/ml; clone 11B11), or anti-IFN- γ (10 μ g/ml; clone XMG1.2). All were obtained from eBioscience (San Diego, CA). Cells were then washed and stained with anti-IFN- γ R α -FITC (clone 2E2; Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-4R α -PE (clone mIL4R-M1; BD Biosciences), and anti-CD4-PE-Cy5 (clone GK1.5; eBioscience).

Real-time PCR

Th1 and Th2 cells differentiated *in vitro* from purified CD4⁺L-selectin⁺ cells were washed, and RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). RNA was quantitated using a spectrophotometer, and the

same amount of RNA from each sample was used to make cDNA and was used as a template for real-time PCR. Each experimental time point sample was used as a template for primer pairs on separate MicroAmp optical 96-well reaction plates using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). To determine the expression ratios between cells induced to differentiate for various time points, all values were normalized to β -actin according to equation: $(Ct_{\text{baseline}} - Ct_{\text{time}})_{\text{YFG}} - (Ct_{\text{baseline}} - Ct_{\text{time}})_{\text{actin}} = \Delta Ct$, then the ratio between treatment and baseline was calculated as $2^{\Delta Ct}$. The sign of the value for ΔCt indicates either up or down-regulation. The primer sequences used were: for Mgat5, 5'-GGAAATGGCCTTGAAAACACA-3' and 5'-CAAGCA CACCTGGGATCCA-3'; and for β -actin 5'-CCAGCAGATGTGGAT CAGCA-3' and 5'-TTGCGGTGCACGATGG-3'. For IFN- γ and IL-4 TaqMan analysis, the murine IFN- γ and IL-4 analysis kit from Applied Biosystems was used.

Results

Regulation of α 1,6GlcNAc-branched *N*-glycan after T cell activation

We have previously shown that T cell activation with anti-CD3 and anti-CD28 Abs significantly increases Mgat5 gene transcription and cell surface β 1,6GlcNAc-branched *N*-glycans (7). The L-PHA binds specifically to mature Mgat5-modified *N*-glycans (20). Staining of Mgat5^{+/+}, Mgat5^{+/-}, and Mgat5^{-/-} resting CD4⁺ T cells with L-PHA-FITC confirmed that Mgat5^{-/-} cells lack specific L-PHA staining (Fig. 1*a*). Mgat5^{+/-} cells had approximately half the staining of wild-type cells, indicating that L-PHA staining is a sensitive and accurate measure of relative differences in Mgat5 *N*-glycan expression and is consistent with our previous finding that Mgat5 enzyme activity in tissues from heterozygous mice is ~50% that in wild-type mice (12). This suggests that Mgat5 enzyme activity is rate-limiting for β 1,6GlcNAc-branched products in T cells. Wild-type T cells were labeled with CFSE to track Mgat5 *N*-glycan expression through each cell division. After stimulation with anti-CD3 with or without anti-CD28 Abs, Mgat5 *N*-glycan expression increased in all cell divisions from 0 to 4, peaking within zero to two cell divisions, then declining modestly with subsequent divisions (Fig. 1, *b-g*). Interestingly, undivided cells in both the CD3- and CD3/CD28-stimulated cultures significantly increased L-PHA staining, indicating that the induction of Mgat5 activity occurs early and does not require cell division. The intensity of L-PHA staining was dependent on the initial stimulus, because increasing the anti-CD3 concentration or adding CD28 both maximized L-PHA staining to ~300% above that in nonstimulated cells (Fig. 1*g*). Because the Mgat5 promoter has Ets and AP-1 binding sites and is responsive to the Ras signaling pathway (21, 22), these data suggest that Mgat5 expression is directly induced by TCR and CD28 signaling. Furthermore, the data indicate that β 1,6GlcNAc-branched *N*-glycan expression is tightly regulated during T cell activation and subsequent cell divisions.

Mgat5 and cytokine production in splenocyte cultures after T cell activation

Splenocytes from Mgat5^{-/-} and wild-type littermates were stimulated with soluble anti-CD3 with or without anti-CD28 for 3 days. Proliferation, measured by [³H]thymidine incorporation, increased in Mgat5^{-/-} spleen cells, compared with that in wild-type cultures (Fig. 2*a*), 4- to 5-fold with anti-CD3 and 2- to 3-fold in anti-CD3 and anti-CD28 cultures. Cytokine production and proliferation index peaked at ~0.5 μ g/ml anti-CD3 Abs for both mutant and wild-type cultures (Fig. 2*b*). Before normalizing for proliferation, Mgat5^{-/-} cultures produced significantly more IFN- γ , IL-2, TNF- α , and IL-10 than wild-type cells. However, after normalizing cytokine production for proliferation, Mgat5^{-/-} cells produced ~2-fold more IFN- γ and ~2-fold less IL-4 on a per cell

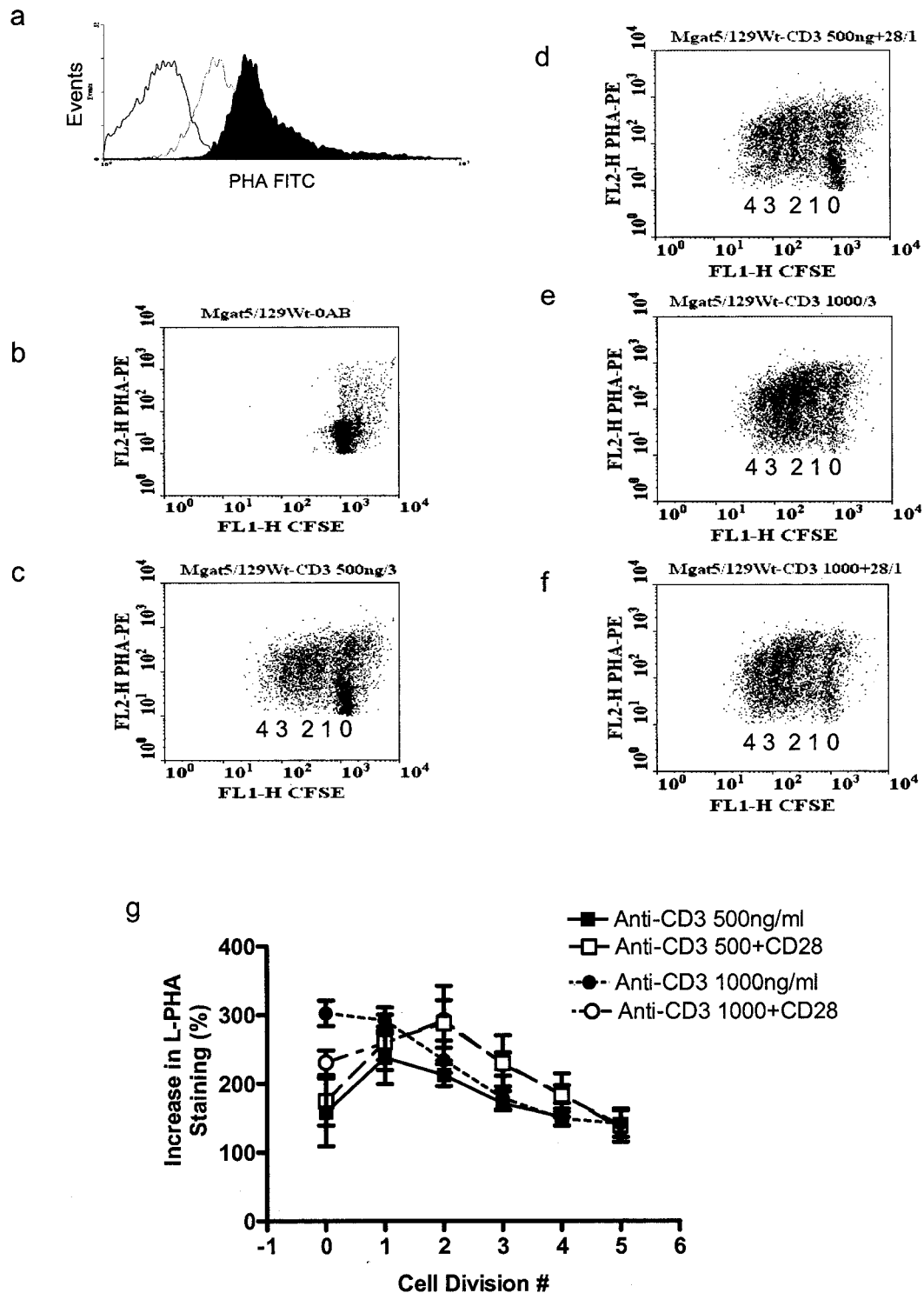


FIGURE 1. Mgat5 modified glycan expression in resting and proliferating CD4⁺ T cells. *a*, Resting splenocytes from Mgat5^{+/+} (■), Mgat5^{+/-} (gray line), and Mgat5^{-/-} (black line) mice were stained with L-PHA-FITC and CD4-PE. L-PHA histograms shown are gated on the CD4-positive population. *b-f*, Purified CD3⁺ T cells from Mgat5^{+/+} mice were labeled with CFSE, cultured for 3 days in the absence (*b*) or the presence of immobilized anti-CD3 ϵ (*c* and *d*, 500 ng/ml; *e* and *f*, 1000 ng/ml) with or without soluble anti-CD28 (*d* and *f*; 1000 ng/ml), stained with L-PHA-PE and anti-CD4-PE-Cy5, and analyzed by FACS. The L-PHA vs CFSE plots shown are gated on the CD4⁺ population. Numbers below the plot represent the number of cell divisions. *g*, L-PHA-FITC mean fluorescence intensity in triplicate for each cell division from *c-f* were averaged, and the percent increase over unstimulated cells (*b*) was plotted vs cell division number.

basis in both anti-CD3- and anti-CD3/anti-CD28-stimulated cultures (Fig. 2*c*). Strikingly, anti-CD3-stimulated Mgat5^{-/-} cells produced markedly more IFN- γ per cell than anti-CD3- and anti-CD28-costimulated wild-type cells. This coupled with the fact that

the addition of anti-CD28 to Mgat5^{-/-} cells did not significantly increase the production of IFN- γ per cell over that of anti-CD3 alone indicates that Mgat5 glycan deficiency sensitizes cells for IFN- γ production much more effectively than CD28 costimulation.

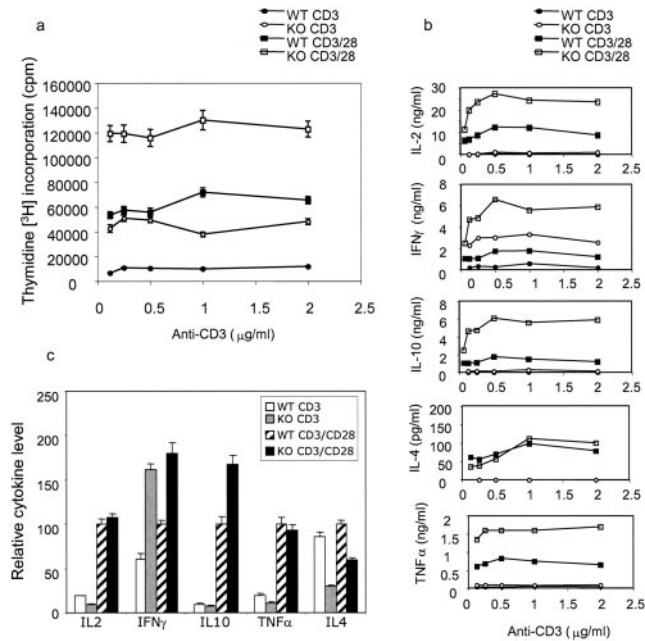


FIGURE 2. Survey of cytokine production in *Mgat5* knockout (KO) splenocytes. *a*, Splenocytes from *Mgat5*^{+/+} and *Mgat5*^{-/-} mice were stimulated with various amounts of soluble anti-CD3 and 1 μ g/ml anti-CD28 for 3 days. Cell proliferation was measured by [³H]thymidine incorporation during the last 20 h of culture. There were no differences in baseline proliferation in KO mice. *b*, Cytokines produced in day 3 supernatant were measured. *c*, Cytokine production from splenocytes stimulated with 0.5 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 described in *a* were normalized for cell proliferation. The ratio of cytokine concentration to counts of thymidine incorporated (cpm) was calculated. The ratio for each cytokine was normalized to 100 for all anti-CD3/anti-CD28-stimulated wild-type samples.

Moreover, CD28 costimulation was unable to compensate for *Mgat5* glycan deficiency in per cell production of IL-4, because anti-CD3- and anti-CD28-costimulated *Mgat5*^{-/-} cells secreted significantly less IL-4 than *Mgat5*^{+/+} cells stimulated with only anti-CD3. An increase in per cell production of IL-10 was also observed in *Mgat5*^{-/-} anti-CD3- and anti-CD28-costimulated cultures (Fig. 2*c*), but, as demonstrated in Fig. 4, this was due to a non-T cell population (see below).

Swainsonine stimulates IFN- γ production in splenocyte cultures

The alkaloid swainsonine is a competitive inhibitor of Golgi α -mannosidase II, which blocks the *N*-glycan biosynthetic pathway before β 1,6GlcNAc branching and results in the production of hybrid-type glycans. Swainsonine is nontoxic in cell culture and has been shown to enhance T cell proliferation and IL-2 production (23). Stimulation of T cells in cultured splenocytes displayed a peak of IFN- γ and IL-4 production on day 3, whereas IL-10 increased until day 5 (Fig. 3*a*). Treatment with swainsonine significantly increased IFN- γ and IL-10 production in cultures stimulated with anti-CD3 Abs alone or combined with anti-CD28 Abs. IFN- γ and IL-10 production reached a maximum with 0.5 μ M swainsonine, whereas cytokine production was reduced by 1 μ M swainsonine (data not shown). Under these conditions, no significant increase in cell number was seen (data not shown).

If swainsonine modifies cytokine production through inhibition of *Mgat5*-modified *N*-glycans, we expect swainsonine treatment and *Mgat5*^{-/-} to be redundant. In this regard, swainsonine treatment of *Mgat5*^{-/-} splenocytes did not generate any further increase in IFN- γ production over that with either stimulant alone

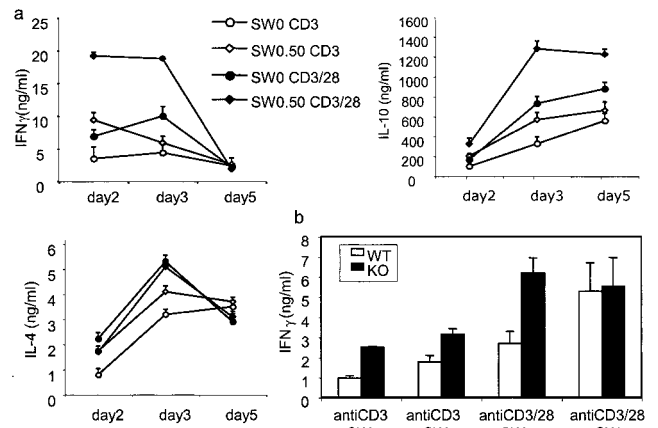


FIGURE 3. Effect of swainsonine on murine cytokine production. *a*, Murine splenocytes were stimulated with plate-bound anti-CD3 with or without 2 μ g/ml anti-CD28 for 5 days. Two concentrations of swainsonine (0 (SW0) and 0.5 (SW0.5) μ g/ml) were included, as indicated in the figure. On days 2, 3, and 5 after stimulation, cytokines in the supernatant were measured. *b*, Swainsonine and *Mgat5* knockout have no additive effect on enhancement of IFN- γ production. Splenocytes from *Mgat5*^{+/+} and *Mgat5*^{-/-} mice were stimulated with plate-bound anti-CD3 with or without soluble anti-CD28 and with or without 0.5 μ g/ml swainsonine for 3 days. IFN- γ production in the supernatant was measured.

(Fig. 3*b*). Thus, both swainsonine and the *Mgat5* mutation enhanced IFN- γ production from splenocytes through the same or similar pathways.

Mgat5 and IFN- γ /IL-4 production by CD4⁺ cells

Because multiple cell types in spleen cell cultures can contribute to cytokine production, we evaluated the contribution by CD4⁺ Th cells alone. *Mgat5* deficiency enhanced IFN- γ and decreased IL-4 production in naive CD4⁺ T cells (CD4⁺CD62L⁺) on a per cell basis when stimulated with plate-bound anti-CD3 and anti-CD28 Abs (Fig. 4). Swainsonine treatment of purified naive CD4 T cells (CD4⁺CD62L⁺) also enhanced IFN- γ production (data not shown). However, no difference between genotypes in IL-10 production per cell was observed in these cultures from days 1–3. On day 6, there was a small decrease in the amount of IL-10 secreted per cell in the *Mgat5*^{-/-} cultures. These data indicate that a non-T cell population contributed to the enhanced IL-10 production in swainsonine-treated and *Mgat5*^{-/-} spleen cell cultures.

To evaluate whether β 1,6GlcNAc-branched *N*-glycans also influence Th1 and Th2 differentiation of human T cells, we examined the effect of swainsonine treatment on cytokine production by human naive T cells. Swainsonine inhibited cell surface L-PHA staining on both CD4⁺ and CD4⁻ T cells purified from peripheral blood in a dose-dependent manner at concentrations of 0.1–0.5 μ M (Fig. 5*a*), and no further inhibition was achieved at 1 μ M (data not shown). Swainsonine inhibited the majority of L-PHA staining, yet a small population of L-PHA-positive cells persisted, suggesting a minor or alternate pathway to complex-type *N*-glycans that uses α -mannosidase IIX is functional in these cells (24). Naive T cells (CD4⁺CD45RO⁻) were stimulated with anti-CD3 and anti-CD28. IL-2 reached peak level on day 2, whereas IFN- γ and IL-10 reached their highest levels 3 days after stimulation (Fig. 5*b*). Swainsonine alone did not stimulate IFN- γ and IL-10 production, but addition of swainsonine with either anti-CD3 Abs or anti-CD3 plus anti-CD28 Abs increased IFN- γ production. IL-10 production was not enhanced by swainsonine treatment. The absolute amount of cytokine production varied among donors, yet swainsonine induced a consistent dose-dependent stimulation of IFN- γ .

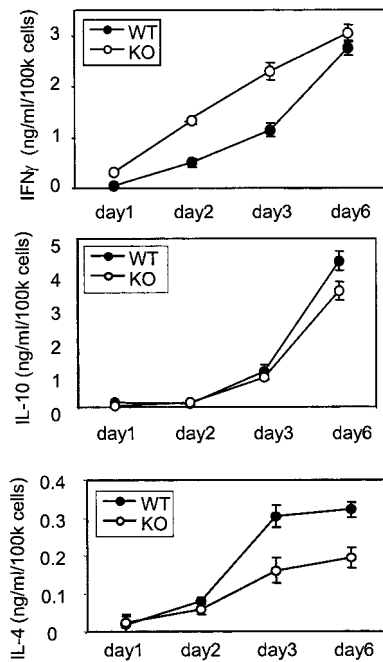


FIGURE 4. Cytokine production in $Mgat5^{+/+}$ and $Mgat5^{-/-}$ naive T cells. $CD4^{+}/CD62L^{+}$ T cells were isolated from the spleens of $Mgat5^{+/+}$ and $Mgat5^{-/-}$ mice and stimulated with plate-bound anti-CD3 and 2 $\mu\text{g}/\text{ml}$ soluble anti-CD28 for 6 days. On days 1, 2, 3, and 6 after stimulation, cytokines in the supernatant were measured. Cell numbers at each time point were recorded, and the amount of cytokine production per 100,000 cells was plotted.

Expression of *Mgat5* and $\beta 1,6\text{GlcNAc}$ -branched N-glycans in Th1 and Th2 cells

Enhancement of IFN- γ and reduced IL-4 production in $Mgat5^{-/-}$ T cells suggest differential roles of $\beta 1,6\text{GlcNAc}$ -branched N-glycans in Th1 and Th2 cells. We therefore examined the L-PHA staining pattern in Th1 and Th2 cells differentiated in vitro. Splenocytes from DO11.10/Rag1 $^{-/-}$ mice were stimulated with Ag under Th1 or Th2 conditions in vitro, and the expression of $\beta 1,6\text{GlcNAc}$ -branched N-glycans on CD4 T cells was examined with L-PHA staining. Naive T cells in the nonstimulated sample were mostly small cells (as shown by forward scatter; Fig. 6a) with low L-PHA staining. L-PHA staining in both Th1 and Th2 cells gradually increased during the early part of the time course and decreased somewhat at the end of the stimulation cycle relative to the peak level. Although no dramatic differences in overall mean fluorescence of L-PHA staining were seen between Th1 and Th2 cells, some differences in the L-PHA staining pattern were apparent. In Th1 cells, the L-PHA staining at each stage was roughly proportional to cell size, i.e., when cells got bigger after several days of stimulation, they acquired more L-PHA staining, whereas this was not the case for Th2 cells (Fig. 6a). To further examine the *Mgat5* gene expression pattern in Th1 and Th2 cells, we performed real-time PCR analysis of *Mgat5* message in purified Th cell populations. Similar to the results from L-PHA staining, on day 4 after TCR stimulation and Th1/Th2 differentiation, a small increase in *Mgat5* expression was seen in Th1 compared with Th2 cells. In this system, Th cells did not express a large quantity of cytokines unless restimulated. To examine *Mgat5* expression during active cytokine expression, we restimulated Th1 and Th2 cells with TCR-stimulating Ab and analyzed *Mgat5* expression at 4 h postrestimulation, when T cells were actively secreting cytokines. A dramatic increase in *Mgat5* expression level was seen in both Th1 and Th2

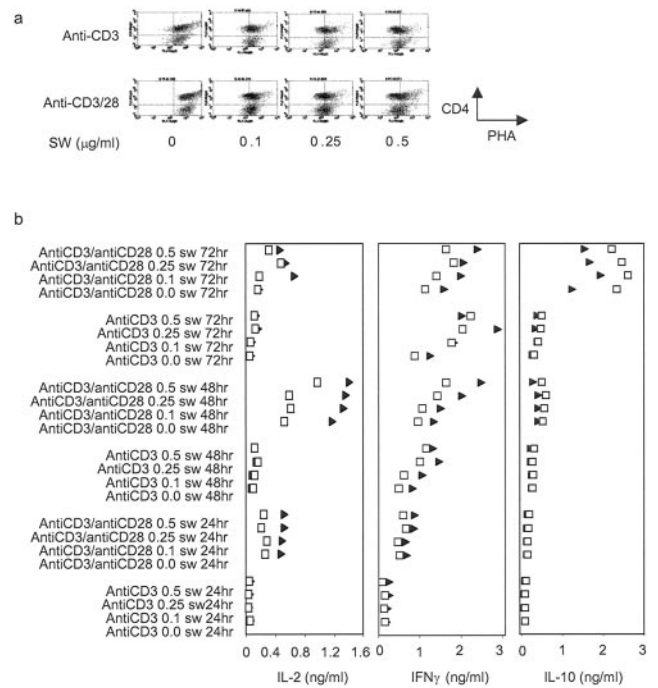


FIGURE 5. Stimulation of IFN- γ production in human T cells by swainsonine. *a*, Inhibition of L-PHA staining of human T cells by swainsonine. Human total T cells ($CD4^{+}$ and $CD8^{+}$) were stimulated with the indicated Abs in the presence of 0, 0.1, 0.25, and 0.5 $\mu\text{g}/\text{ml}$ swainsonine. Three days after stimulation, cells were stained with FITC-conjugated L-PHA (x-axis) and PE-conjugated anti-CD4 Ab (y-axis) and analyzed by FACS. *b*, Cytokine production from primary human T cells upon swainsonine treatment. $CD45RO^{-}$ T cells were isolated from peripheral blood of healthy volunteers, stimulated with anti-CD3 with or without anti-CD28 and the indicated concentrations of swainsonine (micrograms per milliliter) for 3 days. At 24, 48, and 72 h after stimulation, cytokines in the supernatant were measured. Data from donor A (\blacktriangle) and donor B (\square) are shown.

cells upon restimulation, which coincided with high levels of IFN- γ and IL-4 expression in Th1 and Th2 cells, respectively (Fig. 6b). Interestingly, when restimulated, Th1 cells consistently displayed higher *Mgat5* expression than Th2 cells.

Cytokine receptor cell surface expression and *Mgat5*-modified N-glycans

Because greater affinity of TCR-ligand binding can favor the development of the Th1 response, the hypersensitivity of TCR in $Mgat5^{-/-}$ T cells may initiate early events that mediate subsequent feedback regulation of $CD4^{+}$ T cells. To examine $CD4^{+}$ cell responsiveness, as reflected by changes in cell surface receptors, we measured IL-4, IFN- γ , and IL-12 receptors during stimulation. Resting $CD4^{+}$ T cells from $Mgat5^{+/+}$ and $Mgat5^{-/-}$ $CD4^{+}$ T cells displayed similar levels of IL-4R α and IFN- γ R α , whereas no IL-12R $\beta 2$ was detected in either genotype (Fig. 7a and data not shown). After TCR stimulation, surface IL-4R α was down-regulated significantly in $Mgat5^{+/+}$ cells, but decreased only minimally in $Mgat5^{-/-}$ T cells (Fig. 7a). IFN- γ R α levels did not change with stimulation and were unaffected by the *Mgat5* mutation (Fig. 7a). Similarly, IL-12R $\beta 2$ staining was negative in both cells at rest and was similarly increased at 5 days after anti-CD3 stimulation (data not shown). The addition of exogenous IL-4 eliminated surface IL-4R α in both $Mgat5^{+/+}$ and $Mgat5^{-/-}$ cells, indicating that IL-4 cytokine causes a dose-dependent feedback suppression of IL-4R α expression. Confirming this interpretation, anti-IL-4 Ab restored IL-4R α surface expression in $Mgat5^{+/+}$

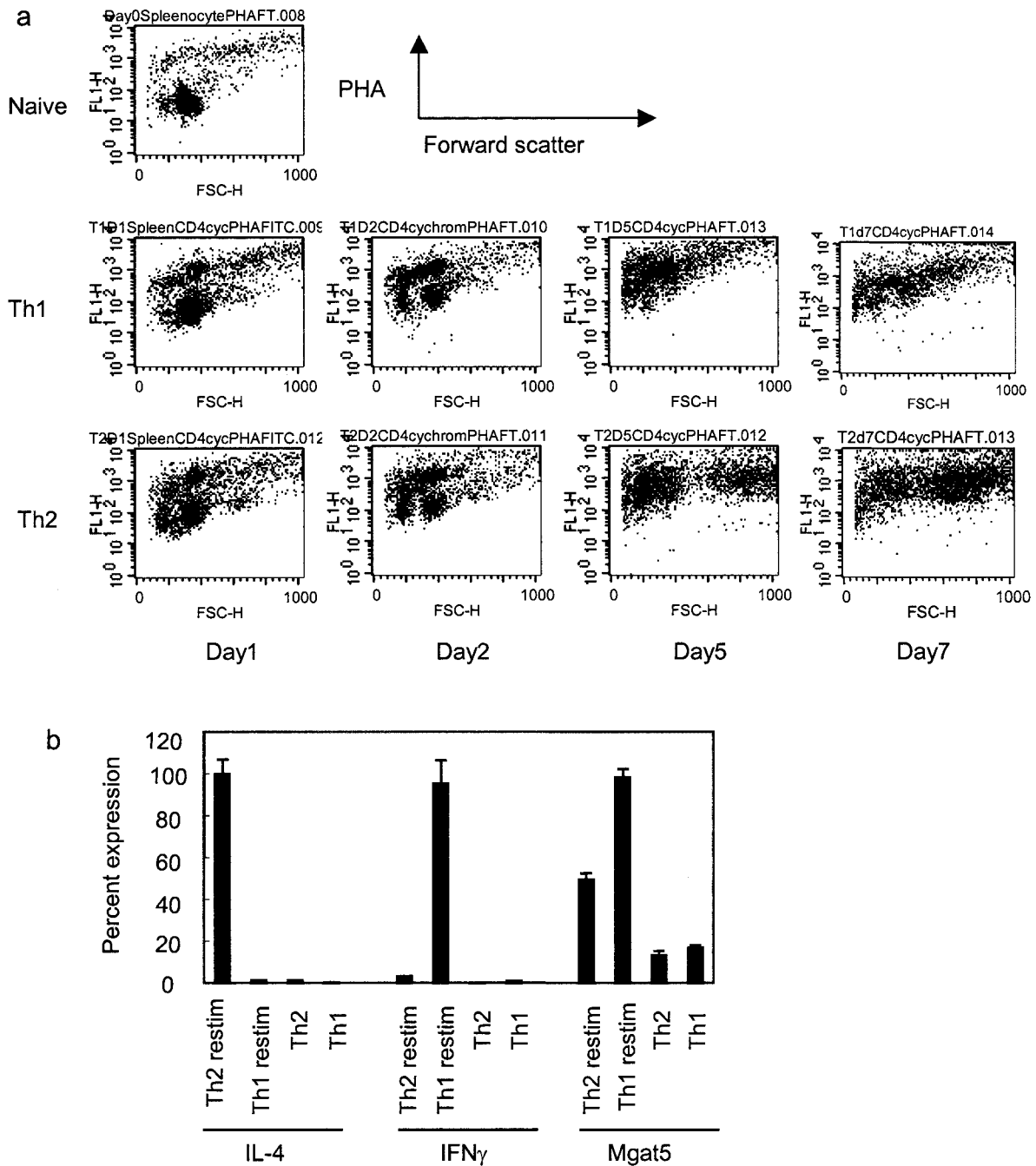


FIGURE 6. L-PHA staining and *Mgat5* mRNA expression in Th1 and Th2 polarized cells. *a*, L-PHA staining in Th1 and Th2 cells. Splenocytes from DO11.10 TCR and *Rag1*^{-/-} double-transgenic mice were stimulated with 300 ng/ml OVA peptide and differentiating cytokines and Abs to differentiate into Th1 and Th2 cells. At various days after stimulation, cells were stained with L-PHA-FITC and analyzed by FACS. Staining of L-PHA (y-axis) and cell size (x-axis; forward scatter) are shown. *b*, *Mgat5* mRNA expression in Th1 and Th2 cells. Purified naive T cells (CD4⁺L-selectin⁺ cells from spleen) were differentiated in vitro using plate-bound anti-CD3, soluble anti-CD28, and polarizing cytokines and Abs into Th1 and Th2 cells. Four days after differentiation, cells were washed and restimulated with soluble anti-CD3 for 4 h. *Mgat5* expression in the restimulated Th1 and Th2 cells was analyzed using SYBR Green real-time PCR. IFN- γ and IL-4 mRNA levels in the same samples were also analyzed. β -Actin was used to normalize the PCR products. Data are presented as the percent expression relative to the highest expression sample.

cells to the same level as in *Mgat5*^{-/-} cells (Fig. 7*b*). Anti-IFN- γ Abs and exogenous IL-12 and IFN- γ had no effect on receptor levels (Fig. 7). Therefore, IL-4R α down-regulation is secondary to increased production of endogenous IL-4, which is reduced in *Mgat5*-deficient T cell cultures. This confirms that *Mgat5* glycans promote IL-4 secretion during T cell activation and demonstrates a functional alteration in *Mgat5*^{-/-} cells.

To determine whether *Mgat5* deficiency can revert an ongoing Th2 response to Th1, we examined cytokine production by polarized Th1 and Th2 cells. The absence of *Mgat5*-modified *N*-glycans did not significantly alter IL-4 production by Th1 and Th2 polarized cells, nor IFN- γ production by Th1 cells (Table I). However, IFN- γ produced by *Mgat5*^{-/-} Th2 polarized cells was ~10-fold higher than that produced by wild-type Th2 cells, although the

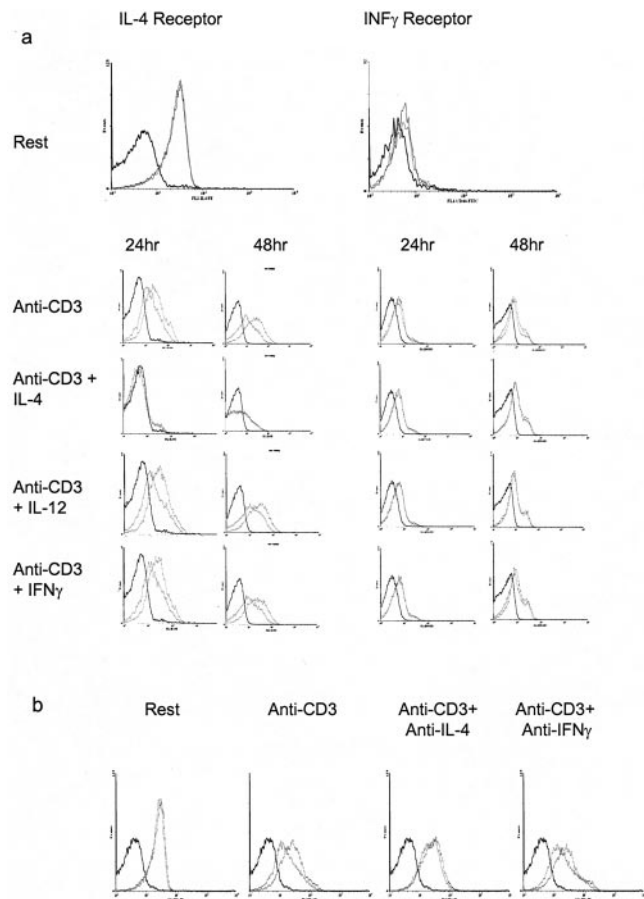


FIGURE 7. FACS analysis of cytokine receptor surface expression in $Mgat5^{+/+}$ and $Mgat5^{-/-}$ $CD4^+$ T cells. *a* and *b*, $CD3^+$ T cells were isolated from the spleens of $Mgat5^{+/+}$ (red) and $Mgat5^{-/-}$ (green) mice, stimulated with plate-bound anti-CD3 (1 μ g/ml) in the presence or the absence of the indicated cytokines and Abs, washed, and then stained for CD4, IL-4R α , and IFN- γ R α . The plots shown are gated on the $CD4^+$ population. Control staining (black) for anti-IL-4R α is the isotype control. For anti-IFN- γ R α staining, control staining is the isotype control for cells at rest and nonstained for stimulated cells.

absolute IFN- γ level was ~ 15 times lower than that of Th1 polarized cells (Table I). These data indicate that $Mgat5$ deficiency does not significantly alter IFN- γ production by Th1 cells under polarizing conditions, but weakens the polarization of Th2 cells upon restimulation.

Discussion

In this study we used a genetic model and chemical inhibition to demonstrate that $\beta 1,6$ GlcNAc-branched *N*-glycans suppress the production of the major Th1 cytokine IFN- γ and promote the expression of the Th2-inducing cytokine IL-4 in TCR-stimulated splenocytes and naive $CD4^+$ T cells. The magnitude of this effect was highly significant, because $Mgat5$ glycan deficiency enhanced IFN- γ /cell production more effectively than CD28 costimulation, and CD28 costimulation was unable to restore the reduced IL-4/cell production by $Mgat5^{-/-}$ cells to that produced by noncostimulated wild-type splenocytes. The reduced IL-4 production by activated $Mgat5^{-/-}$ T cells was associated with decreased ligand-induced IL-4R α down-regulation. In contrast, $\beta 1,6$ GlcNAc-branched *N*-glycans did not significantly alter IFN- γ R α or IL-12R $\beta 2$ expression. Analysis of TCR-stimulated Th1 and Th2 polarized cells demonstrated that $\beta 1,6$ GlcNAc-*N*-glycan defi-

Table I. Cytokine production in Th1 and Th2 polarized cells from $Mgat5^{+/+}$ and $Mgat5^{-/-}$ mice^a

	IFN- γ (ng/ml)	IL-4 (ng/ml)
Th1 ^{+/+}	36 \pm 2.12	0.2 \pm 0.04
Th1 ^{-/-}	39 \pm 2.93	0.16 \pm 0.02
Th2 ^{+/+}	0.2 \pm 0.03	5.1 \pm 0.29
Th2 ^{-/-}	2.3 \pm 0.39	5.0 \pm 0.21

^a Naive $CD4^+$ T cells were isolated from mouse spleens and stimulated with anti-CD3 and anti-CD28 under Th1 or Th2 differentiation conditions as described in *Materials and Methods*. Four days later, cells were restimulated with 1 μ g/ml anti-CD3 overnight and cytokines in the supernatant were measured. The SD is indicated.

ciency enhanced IFN- γ production by polarized Th2 cells ~ 10 -fold, but did not alter the cytokine profile of polarized Th1 cells. Taken together, these data indicate that $Mgat5$ glycans promote the differentiation and enhance the polarization of Th2 cells.

IFN- γ and IL-12 promote Th1, whereas IL-4 enhances Th2 differentiation, respectively. IFN- γ -mediated signaling via the STAT1 signaling pathway induces expression of the transcription factor t-bet, which, in turn, induces expression of IL-12R $\beta 2$ and commits the cell to Th1 differentiation (25, 26). However, Th1/Th2 differentiation by naive $CD4^+$ T cells is also strongly regulated by the potency of the initial TCR signal, because greater ligand affinity or dose promotes IFN- γ production and Th1 differentiation over IL-4 secretion and Th2 lineage commitment (15–18). $Mgat5$ -modified *N*-glycans on TCR regulate TCR association with a multivalent galectin-glycoprotein lattice that restricts ligand-induced TCR recruitment to the immune synapse (7). The absence of $Mgat5$ -modified *N*-glycans weakens the lattice, thereby enhancing agonist-induced TCR clustering, signaling, and proliferation. Taken together these data indicate that deficiency of $\beta 1,6$ GlcNAc-branched *N*-glycans promotes IFN- γ production and Th1 differentiation by directly increasing the strength of primary TCR signals. Thus, differential regulation of $Mgat5$ *N*-glycan expression represents an Ag-independent mechanism to regulate naive $CD4^+$ T cell proliferation and Th1/Th2 differentiation during a primary immune response.

$Mgat5^{-/-}$ mice spontaneously develop late-onset kidney autoimmune disease and are more susceptible to experimental autoimmune encephalomyelitis, the animal model for multiple sclerosis (7). Although not consistent for all types of autoimmunity and experimental systems, IFN- γ and Th1 responses predominantly promote, whereas IL-4 and Th2 responses largely inhibit, autoimmune disease (27). This suggests that the enhanced Th1 differentiation associated with $Mgat5$ deficiency promoted the loss of immune tolerance and autoimmunity observed in null mice. Although resting naive $CD4^+$ T cells continuously engage self-peptide-MHC in the periphery, these interactions normally do not induce significant T cell proliferation or cytokine production, thereby maintaining self-tolerance (28). One likely explanation for this is the failure of self-peptide to activate APCs. Dendritic cells and macrophages express TLRs that recognize pathogen-associated molecular patterns, such as LPS, peptidoglycan, CpG DNA motifs, dsRNA, and bacterial flagellin (29). Recognition of these non-self microbial products by TLRs leads to up-regulation of MHC, CD28 costimulatory receptors B7-1/B7-2, and proinflammatory cytokines, significantly enhancing their ability to stimulate naive $CD4^+$ T cells (29). The inability of self-peptide to trigger these events prevents naive $CD4^+$ T cells from receiving the CD28 and cytokine costimulatory signals necessary for activation. Our previous and current work demonstrates that the increase in T cell

proliferation and IFN- γ production associated with Mgat5 deficiency is the same or greater than that produced by CD28 costimulation. Thus, in the appropriate *in vivo* setting, Mgat5^{-/-} naive CD4⁺ T cells may proliferate and secrete IFN- γ in response to self-peptide-MHC despite presentation by unactivated or minimally activated APC. This may be sufficient to promote a Th1 response to self-peptide, the breakdown of self-tolerance, and autoimmunity.

β 1,6GlcNAc-branched *N*-glycans on TCR bind galectins, forming a multivalent lattice that inhibits TCR recruitment into the immune synapse (7), indicating an inhibitory role for β 1,6GlcNAc-branched *N*-glycans in TCR signaling. After stimulation, Mgat5 glycan expression increases \sim 300% within the first few cell divisions, then slowly declines. This suggests that Mgat5 acts early to limit further TCR stimulation. However, Mgat5 glycans also negatively regulate adhesion to fibronectin in cancer cells and fibroblasts, a function we have also observed in T cells (M. Demetriou and J. W. Dennis, unpublished observations). This raises the possibility that the increased expression of Mgat5 glycans early in the T cell proliferation cycle acts to reduce adhesion to fibronectin, thereby promoting departure from the site of Ag presentation in lymphoid tissues and recruitment to sites of infection/inflammation.

Mgat5 and β 1,6GlcNAc-branched *N*-glycan expression in differentiating Th1 and Th2 cells were generally similar; however, when resting cells are restimulated via TCR, Th2 cells induce significantly less Mgat5 mRNA than Th1 cells. Th2 cells have reduced recruitment of TCR to the site of Ag presentation compared with Th1 cells, resulting in reduced TCR signaling events, such as calcium mobilization and tyrosine phosphorylation of Zap-70 and Fyn (30–32). Our finding that the strength of TCR stimulation regulates Mgat5 glycan expression (Fig. 1) is consistent with the reduced TCR clustering and signaling present in Th2 cells limiting Mgat5 mRNA up-regulation. Although the functional significance of this differential regulation is not clear, it may result in alterations in subsequent TCR activation and/or adhesion to fibronectin, both of which possibilities require further investigation.

IL-10 was increased in Mgat5^{-/-} splenocyte cultures, but not in purified T cells, suggesting that non-T cell populations contribute directly or indirectly to differential cytokine production. Macrophages are major producers of IL-10, and other sources of IFN- γ include CD8 T cells and NK cells. A recent study showed that M150, a membrane protein on activated macrophages that promotes Th1 differentiation of naive T cells, is a uniquely glycosylated form of lysosomal-associated membrane protein-1 (33). Lysosomal-associated membrane protein-1 contains 20 Asn-linked glycosylation sites and is a major target for β 1,6GlcNAc branching in cell lines with increased Mgat5 expression (34–36), suggesting that Mgat5 deficiency in macrophages may alter the ability of M150 to drive Th1 differentiation.

Mgat5 gene transcription is positively regulated by Ras-Raf-Ets (21, 22), a pathway commonly activated in cancer cells as well as activated T cells. Mgat5-modified glycans in human breast and colorectal carcinomas correlate with progression (34) and reduced patient survival time (37). Transfection of mouse mammary carcinoma cells with an Mgat5 expression vector enhances invasion and metastasis (38, 39). Conversely, tumor cell mutants selected for L-PHA resistance and found to be deficient in Mgat5 activity are also deficient for metastasis (40). In a transgenic model of mammary carcinoma, Mgat5 deficiency is associated with significantly reduced tumor growth and metastasis (12). These experiments suggest that Mgat5 regulates tumor cell autonomous phenotypes, but does not exclude the possibility that enhanced T cell activation in Mgat5^{-/-} mice also contributes to reduced tumor

progression. For instance, the expression of a dominant negative form of TGF- β receptor in T cells is sufficient to induce immune rejection in wild-type tumors (41). The results presented in this study demonstrate enhanced Th1 cytokine production in Mgat5^{-/-} T cells, suggesting that enhanced Th1-mediated antitumor immunity may also play a role in tumor reduction in Mgat5^{-/-} mice.

Swainsonine is a potent low m.w. inhibitor of α -mannosidase II, an enzyme upstream of Mgat5 in the *N*-glycosylation pathway. Swainsonine has been shown to inhibit tumor growth and lung colonization in a number of animal models (42–44) and has demonstrated low toxicity as well as evidence of clinical efficacy in two phase I clinical trials (45). In addition to its direct effect on tumor cells, swainsonine enhances activation of Th cells (46), CTLs (47), NK and LAK cells (23, 48), as well as macrophages (49), all of which have potential for antitumor activity. In this study we show that swainsonine enhances IFN- γ production, the key Th1 cytokine that stimulates CD8 cells and NK cells, the major effector cells involved in tumor eradication. Previous publications have shown that swainsonine has no effect on IFN- γ production by murine splenocytes (39). However, these researchers used a much higher concentration of swainsonine. We found that the peak level of IFN- γ stimulation occurs at \sim 0.5–1 μ M swainsonine, with IFN- γ production leveling off when higher concentrations of swainsonine were used. Higher levels of swainsonine inhibit lysosomal α -mannosidases and cause high mannose storage, which may interfere with immune cell functions. Swainsonine might be considered a lead compound, and with a growing appreciation of its mechanism of action, an analog with improved specificity could be useful in cancer therapy.

References

- Daniels, M. A., K. A. Hogquist, and S. C. Jameson. 2002. Sweet 'n' sour: the impact of differential glycosylation on T cell responses. *Nat. Immunol.* 3:903.
- Siegelman, M. H., H. C. DeGrendele, and P. Estess. 1999. Activation and interaction of CD44 and hyaluronan in immunological systems. *J. Leukocyte Biol.* 66:315.
- Blander, J. M., I. Visintin, C. A. Janeway, Jr., and R. Medzhitov. 1999. α (1,3)-fucosyltransferase VII and α (2,3)-sialyltransferase IV are up-regulated in activated CD4 T cells and maintained after their differentiation into Th1 and migration into inflammatory sites. *J. Immunol.* 163:3746.
- Lim, Y. C., H. Xie, C. E. Come, S. I. Alexander, M. J. Grusby, A. H. Lichtman, and F. W. Luscinskas. 2001. IL-12, STAT4-dependent up-regulation of CD4⁺ T cell core 2 β -1,6-N-acetylglucosaminyltransferase, an enzyme essential for biosynthesis of P-selectin ligands. *J. Immunol.* 167:4476.
- Kaufmann, M., C. Blaser, S. Takashima, R. Schwartz-Albiez, S. Tsuji, and H. Pircher. 1999. Identification of an α 2,6-sialyltransferase induced early after lymphocyte activation. *Int. Immunol.* 11:731.
- Austrup, F., D. Vestweber, E. Borges, M. Lohning, R. Brauer, U. Herz, H. Renz, R. Hallmann, A. Scheffold, A. Radbruch, and A. Hamann. 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. *Nature* 385:81.
- Demetriou, M., M. Granovsky, S. Quaggin, and J. W. Dennis. 2001. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 409:733.
- Cummings, R. D., and S. Kornfeld. 1984. The distribution of repeating Gal b1-4GlcNAc b1-3 sequences in asparagine-linked oligosaccharides of the mouse lymphoma cell line BW5147 and PHAR 2.1. *J. Biol. Chem.* 259:6253.
- Yousefi, S., E. Higgins, Z. Daoling, A. Pollex-Kruger, O. Hindsgeul, and J. W. Dennis. 1991. Increased UDP-GlcNAc:Gal b1-3GalNAc-R (GlcNAc to GalNAc) b1-6 N-acetylglucosaminyltransferase activity in transformed and metastatic murine tumor cell lines: control of polyactosamine-synthesis. *J. Biol. Chem.* 266:1772.
- Hubbard, S. C., D. M. Kranz, G. D. Longmore, M. V. Sitkovsky, and H. N. Eisen. 1986. Glycosylation of the T-cell antigen-specific receptor and its potential role in lectin-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA* 83:1852.
- Ahmad, N., H. J. Gabius, S. Andre, H. Kaltner, S. Sabesan, R. Roy, B. Liu, F. Macaluso, and C. F. Brewer. 2003. Galectin-3 binds as a pentamer to synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. *J. Biol. Chem.* 279:10841.
- Granovsky, M., J. Fata, J. Pawling, W. J. Muller, R. Khokha, and J. W. Dennis. 2000. Suppression of tumor growth and metastasis in Mgat5 deficient mice. *Nat. Med.* 6:306.
- Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145.

14. Seder, R. P., and W. E. Paul. 1994. Acquisition of lymphokine-producing phenotype by CD4⁺ T cells. *Annu. Rev. Immunol.* 12:635.
15. Hosken, N. A., K. Shibuya, A. W. Heath, K. M. Murphy, and A. O'Garra. 1995. The effect of antigen dose on CD4⁺ T helper cell phenotype development in a T cell receptor- α β -transgenic model. *J. Exp. Med.* 182:1579.
16. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4⁺ T cells. *J. Exp. Med.* 182:1591.
17. Tao, X., S. Constant, P. Jorritsma, and K. Bottomly. 1997. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4⁺ T cell differentiation. *J. Immunol.* 159:5956.
18. Ise, W., M. Totsuka, Y. Sogawa, A. Ametani, S. Hachimura, T. Sato, Y. Kumagai, S. Habu, and S. Kaminogawa. 2002. Naive CD4⁺ T cells exhibit distinct expression patterns of cytokines and cell surface molecules on their primary responses to varying doses of antigen. *J. Immunol.* 168:3242.
19. Li, B., C. Tournier, R. J. Davis, and R. A. Flavell. 1999. Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. *EMBO J.* 18:420.
20. Cummings, R. D., and S. Kornfeld. 1982. Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized *Phaseolus vulgaris* leucoagglutinating and erythroagglutinating lectin. *J. Biol. Chem.* 257:11230.
21. Chen, L., W. Zhang, N. Fregien, and M. Pierce. 1998. The her-2/neu oncogene stimulates the transcription of *N*-acetylglucosaminyltransferase V and expression of its cell surface oligosaccharide products. *Oncogene* 17:2087.
22. Ko, J. H., E. Miyoshi, K. Noda, A. Ekuni, R. Kang, Y. Ikeda, and N. Taniguchi. 1999. Regulation of the GnT-V promoter by transcription factor Ets-1 in various cancer cell lines. *J. Biol. Chem.* 274:22941.
23. Bowlin, T. L., B. J. McKown, M. S. Kang, and P. S. Sunkara. 1989. Potentiation of human lymphokine-activated killer cell activity by swainsonine, an inhibitor of glycoprotein processing. *Cancer Res.* 49:4109.
24. Chui, D. M. Oh-Eda, Y. F. Liao, K. Panneerselvam, A. Lal, K. W. Marek, H. H. Freeze, K. W. Moremen, M. N. Fukuda, and J. D. Marth. 1997. α -Mannosidase-II deficiency results in dyserythropoiesis and unveils an alternate pathway in oligosaccharide biosynthesis. *Cell* 90:157.
25. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655.
26. Afkarian, M., J. R. Sedy, J. Yang, N. G. Jacobson, N. Cereb, S. Y. Yang, T. L. Murphy, and K. M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4⁺ T cells. *Nat. Immunol.* 3:549.
27. O'Shea, J. J., A. Ma, and P. Lipsky. 2002. Cytokines and autoimmunity. *Nat. Rev. Immunol.* 2:37.
28. Jameson, S. 2002. Maintaining the norm: T-cell homeostasis. *Nat. Rev. Immunol.* 2:547.
29. Barton, G. M., and R. Medzhitov. 2002. Control of adaptive immune responses by Toll-like receptors. *Curr. Opin. Immunol.* 14:380.
30. Gajewski, T. F., S. R. Schell, and F. W. Fitch. 1990. Evidence implicating utilization of different T cell receptor-associated signaling pathways by TH1 and TH2 clones. *J. Immunol.* 144:4110.
31. Sloan-Lancaster, J., T. H. Steinberg, and P. M. Allen. 1997. Selective loss of the calcium ion signaling pathway in T cells maturing toward a T helper 2 phenotype. *J. Immunol.* 159:1160.
32. Tamura, T., H. Nakano, H. Nagase, T. Morokata, O. Igarashi, Y. Oshimi, S. Miyazaki, and H. Nariuchi. 1995. Early activation signal transduction pathways of Th1 and Th2 cell clones stimulated with anti-CD3: roles of protein tyrosine kinases in the signal for IL-2 and IL-4 production. *J. Immunol.* 155:4692.
33. Prasad, D. V., V. V. Parekh, B. N. Joshi, P. P. Banerjee, P. B. Parab, S. Chattopadhyay, A. Kumar, and G. C. Mishra. 2002. The Th1-specific costimulatory molecule, m150, is a posttranslational isoform of lysosome-associated membrane protein-1. *J. Immunol.* 169:1801.
34. Dennis, J. W., S. Laferte, C. Waghome, M. L. Breitman, and R. S. Kerbel. 1987. β 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science* 236:582.
35. Laferte, S., and J. W. Dennis. 1989. Purification of two glycoproteins expressing β 1-6 branched Asn-linked oligosaccharides from metastatic tumour cells. *Biochem. J.* 259:569.
36. Demetriou, M., I. R. Nabi, M. Coppolino, S. Dedhar, and J. W. Dennis. 1995. Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. *J. Cell Biol.* 130:383.
37. Dennis, J. W., and Laferte, S. 1989. Oncodevelopmental expression of -GlcNAc β 1-6Man α 1-6Man β 1-branched asparagine-linked oligosaccharides in murine tissues and human breast carcinomas. *Cancer Res.* 49:945.
38. Balamuth, F., D. Leitenberg, J. Unternaehrer, I. Mellman, and K. Bottomly. 2001. Distinct patterns of membrane microdomain partitioning in Th1 and th2 cells. *Immunity* 15:729.
39. Kosuge, T., T. Tamura, H. Nariuchi, and S. Toyoshima. 2000. Effect of inhibitors of glycoprotein processing on cytokine secretion and production in anti CD3-stimulated T cells. *Biol. Pharm. Bull.* 23:1.
40. Dennis, J. W., C. E. Warren, M. Granovsky, and M. Demetriou. 2001. Genetic defects in *N*-glycosylation and cellular diversity in mammals. *Curr. Opin. Struct. Biol.* 11:601.
41. Gorelik, L., and R. A. Flavell. 2001. Immune-mediated eradication of tumors through the blockade of transforming growth factor- β signaling in T cells. *Nat. Med.* 7:1118.
42. Dennis, J. W. 1986. Effects of swainsonine and polyinosinic:polycytidylic acid on murine tumor cell growth and metastasis. *Cancer Res.* 46:5131.
43. Dennis, J. W., K. Koch, and D. Beckner. 1989. Inhibition of human HT29 colon carcinoma growth in vitro and in vivo by swainsonine and human interferon- α 2. *J. Natl. Cancer Inst.* 81:1028.
44. Humphries, M. J., K. Matsumoto, S. L. White, R. J. Molyneux, and K. Olden. 1990. An assessment of the effects of swainsonine on survival of mice injected with B16-F10 melanoma cells. *Clin. Exp. Metastasis* 8:89.
45. Goss, P. E., M. A. Baker, J. P. Carver, and J. W. Dennis. 1995. Inhibitors of carbohydrate processing: a new class of anticancer agents. *Clin. Cancer Res.* 1:935.
46. Bowlin, T. L., and P. S. Sunkara. 1988. Swainsonine, an inhibitor of glycoprotein processing, enhances mitogen induced interleukin 2 production and receptor expression in human lymphocytes. *Biochem. Biophys. Res. Commun.* 151:859.
47. Yagita, M., and E. Saksela. 1990. Swainsonine, an inhibitor of glycoprotein processing, enhances cytotoxicity of large granular lymphocytes. *Scand. J. Immunol.* 31:275.
48. Galustian, C., S. Foulds, J. F. Dye, and P. J. Guillou. 1994. Swainsonine, a glycosylation inhibitor, enhances both lymphocyte efficacy and tumour susceptibility in LAK and NK cytotoxicity. *Immunopharmacology* 27:165.
49. Grzegorzewski, K., S. A. Newton, S. K. Akiyama, S. Sharrow, K. Olden, and S. L. White. 1989. Induction of macrophage tumoricidal activity, major histocompatibility complex class II antigen (Iak) expression, and interleukin-1 production by swainsonine. *Cancer Commun.* 1:373.