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Review

UDP-*N*-acetylglucosamine:α-6-D-mannoside β1, 6 *N*-acetylglucosaminyltransferase V (*Mgat5*) deficient mice

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Abstract

Targeted gene mutations in mice that cause deficiencies in protein glycosylation have revealed functions for specific glycans structures in embryogenesis, immune cell regulation, fertility and cancer progression. UDP-*N*-acetylglucosamine:α-6-D-mannoside β1,6 *N*-acetylglucosaminyltransferase V (GlcNAc-TV or Mgat5) produces *N*-glycan intermediates that are elongated with poly *N*-acetyllactosamine to create ligands for the galectin family of mammalian lectins. We generated Mgat5-deficient mice by gene targeting methods in embryonic stem cells, and observed a complex phenotype in adult mice including susceptibility to autoimmune disease, reduced cancer progression and a behavioral defect. We found that Mgat5-modified *N*-glycans on the T cell receptor (TCR) complex bind to galectin-3, sequestering TCR within a multivalent galectin–glycoprotein lattice that impedes antigen-dependent receptor clustering and signal transduction. Integrin receptor clustering and cell motility are also sensitive to changes in Mgat5-dependent *N*-glycosylation. These studies demonstrate that low affinity but high avidity interactions between *N*-glycans and galectins can regulate the distribution of cell surface receptors and their responsiveness to agonists.

Keywords: *N*-glycan; Cancer; Immunity; Mgat5; Poly *N*-acetyllactosamine

1. Introduction

N-linked glycans are found on cell surface and secreted proteins that control proliferation, cell fate decisions, and specialized functions in animals. However, the Golgi *N*-glycan processing pathway generates great structural heterogeneity on mature glycoproteins, the purpose of which has been largely unclear. Many glycosyltransferases in the medial- and trans-Golgi compete for acceptor intermediates causing bifurcations of the biosynthesis pathway [1]. In addition, the protein environment of each Asn-X-Ser/Thr site influences access by processing enzymes, and inefficiencies at this level results in only a fraction of *N*-glycans receiving certain substitutions [2]. Therefore, each Asn-X-Ser/Thr site of a glycoprotein is commonly populated by a set of biosynthetically related *N*-glycan structures. Functional properties can vary between glycoforms, with the overall potency of a glycoprotein being the weighted-average of the specific activities of glycoforms in the molecular population (Fig. 1). Consequently, this structural heterogeneity has made it difficult to ascribe functionality to specific *N*-glycans. However, targeted germline mutations affecting *N*-glycan biosynthesis can provide a means of assessing the consequences of removing subsets of structures in the context of the whole animal. Further analysis of organ-specific and cellular phenotypes associated with the mutations can lead to a definition of the molecular pathways and glycoproteins that require these *N*-glycans. In most instances, mice with targeted gene mutations in late stage Golgi

Abbreviations: TCR, T cell receptor; PyMT, polyoma virus middle T oncogene; APC, antigen-presenting cell; Peptide-MHC, antigenic peptide bound to major histocompatibility complex

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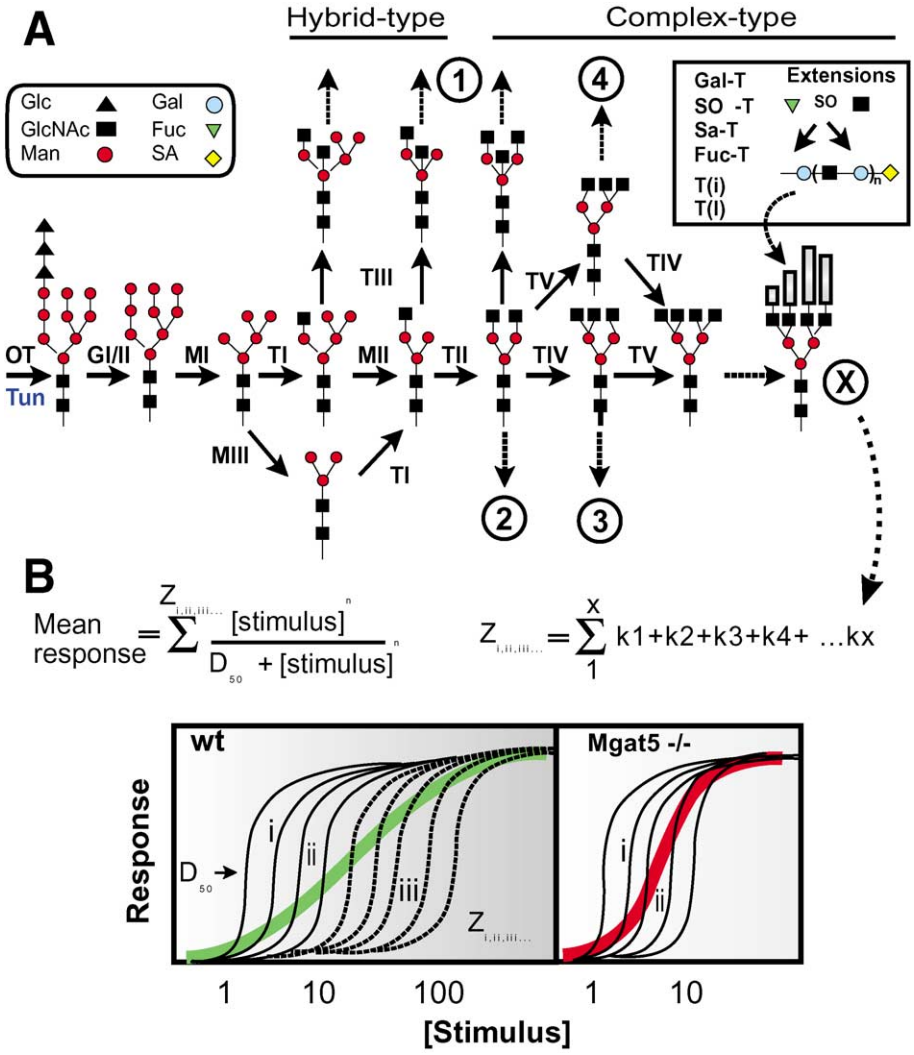

Fig. 1. Glycosylation and plasticity in the T cell population. (A) Scheme of the *N*-glycan biosynthesis pathway. Abbreviations used are oligosaccharyltransferase, OT; the α-glucosidases, GI, GII; the β-*N*-acetylglucosaminyltransferases, TI, TII, TIII, TIV, TV, T(i); the α1,2mannosidases, MI; α1,3/6mannosidases, MII, MIII; β1,4-galactosyltransferases, Gal-T; α-fucosyltransferases, Fuc-T; α-sialyltransferases, SaT; sulfotransferases, SO4-T. Gene names for TI to TV are Mgat1 to Mgat5, respectively. Tun, tunicamycin. The circled numbers 1 to *x* represent biosynthetically related subsets of glycans labeled here only to illustrate the model shown below. (B) Hypothetical model to represent variability or plasticity within the T cell population. For each T cell, entry into S phase is a switch-like event that initiates activation and several rounds of cell division. Each black line represents individual cells or groups of cells that share a similar *N*-glycan structural profile. The weighted-contributions of glycoforms (k_1–k_x) contribute to the cell phenotype (Z_i,), and in particular the response to agonist. The mean response to agonists is the sum of responses for the population overall Z_i. The dashed lines depict the influence of Mgat5-modified glycans. The mean response has a D_{50} and Hill slope as depicted by the colored line for wild type (wt) and Mgat5-deficient mice. Note that the variance in the cell population is largest for wt and reduced in the mutants.

processing enzymes are viable, but display tissue-specific defects [3]. For example, α-mannosidase IIx-deficient male mice are largely infertile due to a failure of spermatocytes adhesion to Sertoli support cells via carbohydrates, and this presumably results in premature release of the cells from the testis to epididymis [4]. The sialytransferase ST6Gal produces a ligand for CD22, a transmembrane lectin recruited into the B cell receptor complex where it dampens the response [5]. Consistent with this role, CD22-deficient mice are hypersensitive to antigen stimulation, while ST6Gal-deficient mice display impaired B cell proliferation and attenuated antibody production [6].

2. Magt5 and expression of β1,6GlcNAc-branched *N*-glycans

Mgat5 transfers GlcNAc from UDP-GlcNAc to the OH on carbon 6 of α-linked Man in the minimal acceptor sequence GlcNAcβ1,2Manα1,6Manβ1, a sequence present in *N*-glycan intermediates at the medial Golgi stage of glycoprotein maturation [7]. Following substitutions by GlcNAc-TV, glycoproteins destined for the cell surface pass through the trans-Golgi where a variety of terminal sequences are added to complete the tri (2,2,6) and tetra (2,4,2,6) antennary complex-type *N*-glycans. The termini include

various combinations of *N*-acetyllactosamine and poly *N*-acetyllactosamine capped with sialic acid, fucose and sulfate. *Mgat5* gene expression is regulated in a tissue-specific manner, and plays an important role in expression of these termini. Notably, the tri (2,2,6) and tetra (2,4,2,6) antennary glycan intermediates produced by Mgat5 are preferred substrates for addition of poly *N*-acetyllactosamine by β1,3GlcNAc-T(i) and β1,4Gal-TI [8,9]. Structural analysis of glycans in mutant tumor cell lines that lack Mgat5 activity revealed a deficiency in addition of poly *N*-acetyllactosamine on *N*-glycans [10,11]. However, poly *N*-acetyllactosamine levels are also dependent upon the activity of extension enzymes β1,4Gal-T and β1-3GlcNAcT(i) {{245}}.

A single gene encodes Mgat5 enzyme activity in mammals and *C. elegans.* The human and rat Mgat5 proteins are 740 amino acids in length and share 97% identity [12,13]. The amino acid sequence $S_{213-740}$ is essential for GlcNAc-TV catalytic activity, leaving a 183 amino acid stem region, a transmembrane domain, and 12 amino acids in the cytosol. Deletion of only five amino acids from the C-terminal end is sufficient to destroy catalytic activity [14]. The *C. elegans* gene designated *gly-2* is 36.7% identical to mammalian Mgat5, has a similar minimal catalytic domain, exon–intron structure and the gene product substitutes the GlcNAc β1,2Manα1,6Manβ1,4GlcNAc-R acceptor in vitro. More importantly, *gly-2* is a functional homologue of *Mgat5*, as it complements the Chinese hamster ovary (CHO) Lec4 mutation by restoring wild type levels of L-PHA reactive glycans at the cell surface [15].

Tissue-specific patterns of gene expression can often provide important clues to gene function. *Mgat5* gene expression is low in the mouse embryo at day 7 of gestation (E7), but widely expressed by E9.5. In late-stage embryos, *Mgat5* message is present in the central nervous system, and basal layers of specialized epithelia of skin, intestine, kidney, endocrine tissues and respiratory tract. With a *gly-2*::GFP-reporter gene in transgenic worms, we also observed high expression in many neuronal cells, as well as spermathecal and pharyngeal–intestinal vulval cells [15]. *Mgat5* transcription is positively regulated by Ras-Raf-Ets in cultured fibroblast cells [16,17], a pathway that is commonly activated in cancer cells. Indeed, early interest in Mgat5 stems from observations that complex-type *N*-glycan structures are commonly larger following malignant transformation [18], and this is due to greater β1,6GlcNAc-branching and poly *N*-acetyllactosamine content [19]. Mgat5 activity increases 2–10-fold in cells transformed by polyoma virus or Rous sarcoma virus [20], and in cells transfected with activated K-Ras [21]. Forced expression of Mgat5 in MuLv1 epithelial cells resulted in loss of contact inhibition, increased cell motility, morphological transformation, and tumor formation in athymic nude mice [22]. Transfection of mouse mammary carcinoma cells with Mgat5 enhances invasion and metastasis [23,24]. Conversely tumor cell mutants selected for L-PHA-resistance and found to be deficient in Mgat5 activity are also deficient for metastasis [21,25]. These experiments suggest that Mgat5 regulates tumor cell autonomous phenotypes.

The plant lectin leukoagglutinin (L-PHA) binds preferentially to the Galβ1-4GlcNAcβ1-6[Galβ1-4GlcNAcβ1-2]Man sequence in mature *N*-glycans [26], and has been used as a probe for Mgat5-modified glycans. L-PHA histochemistry has revealed increased Mgat5-modified glycans in human breast and colorectal carcinomas [27]. High expression of Mgat5 and its *N*-glycan product was observed in human hepatocellular carcinoma and liver cirrhosis but not in normal liver [28]. L-PHA reactivity in primary colorectal cancers correlated with poor prognosis and reduced patient survival time [29]. L-PHA reactivity was more intense at the outer edge of human esophageal squamous-cell carcinomas, suggesting an association with invasive cells, which are expected to be at the margins of the tumor [30].

3. Magt5-deficient mice

To assess the requirement for *Mgat5* in the immune system and cancer progression, we disrupted the *Mgat5* gene in mice using a gene-targeting vector where the first exon was replaced with the β-galactosidase (LacZ) reporter gene. Two independent ES clones that had undergone homologous recombination were used to produce mice, and both were viable strains with identical T cell phenotypes. The tissue distribution of LacZ activity in $Mgat5^{+/-}$ and $Mgat5^{-/-}$ mice was similar to that of the *Mgat5* transcripts in wild type mice, indicating that the reporter gene faithfully reflected *Mgat5* transcription [31]. The *Mgat5* knockout mice appeared normal at birth, and lacked detectable Mgat5 enzyme activity and L-PHA reactive glycoproteins. Adult $Mgat5^{-/-}$ mice differed in their responses to various extrinsic conditions [32], including cancer progression, T cell hypersensitivity, autoimmune disease, and nurturing responses following birth. Regarding the latter phenotype, $Mgat5^{-/-}$ females on the CD1 and C57B/6 strain backgrounds nurtured their pups, while $Mgat5^{-/-}$ mothers on the 129 and PLJ backgrounds often failed to gather, nest and feed their pups in the first day (Pawling, Granovsky, Demetriou, unpublished observations). This appears to be a behavioral defect as the presence of surrogate wild type or heterozygous mothers prevents the death of pups delivered by $Mgat5^{-/-}$ mothers. This phenotype was more penetrant on the PLJ and 129 mouse backgrounds compared to the B57Bl/6 strain, suggesting the existence of strain-dependent modifier genes for this Mgat5 phenotype. Nurturing behavior in mice requires a complex mixture of olfactory, hormonal and visual stimulation, one or more of which may be delayed or lacking in the $Mgat5^{-/-}$ mice.

4. Mgat5 regulates thresholds for TCR activation

A functional role for Mgat5-modified glycans has been suggested by reports that their depletion with swainsonine-treatment, an inhibitor of α-mannosidase II, enhances antigen-dependent T cell proliferation [33,34]. Furthermore, mice deficient in α-mannosidase II also develop autoimmune kidney disease [35]. *Mgat5*$^{-/-}$ mice display an age-related spontaneous autoimmune disease, characterized by glomerulonephritis [36]. Delayed type hypersensitivity skin reactions were more severe, and susceptibility to experimental autoimmune encephalomyelitis (EAE), a model for human multiple sclerosis, was greater in mutant mice. The EAE model is a T cell dependent disease [37] initiated by reactivity against brain proteins, notably myelin basic protein (MBP) and proteolipid apoprotein. The severity of EAE is dependent on the initiating doses of antigenic protein. Significantly, *Mgat5*$^{-/-}$ mice were hypersensitive to low-dose MBP, while high-dose MBP induced disease in 100% of the mutant and wild type animals. Therefore, we examined the effect of the Mgat5 mutation on agonist threshold for T cells stimulation in vitro. Concordant with the in vivo data, we observed that *Mgat5*$^{-/-}$ T cells were hypersensitive to anti-T cell receptor (TCR) antibodies, displaying a lower D_{50} and more cooperative or synchronous proliferation response. Although B cells express L-PHA-reactive *N*-glycans, *Mgat5*$^{-/-}$ B cells responded normally to a variety of stimuli.

The TCRα and β chains have seven *N*-glycans, and CD3 γ and δ each have one chain. A fraction of these glycans are L-PHA reactive, apparently bearing Mgat5-modified complex-type structures [38]. *N*-glycans protrude ~ 30 Å from the protein surface and more, depending upon the number of repeats in polylactosamine sequences. Rudd et al. [39] have modeled biantennary complex-type *N*-glycans onto TCR complex, and suggest that their size and positions on the proteins limits nonspecific receptor aggregation in the plane of the membrane. Additionally, multivalent lectins bind *N*-glycan at the cell surface and may restrict glycoprotein mobility by forming an organized matrix or planar lattice over the membrane. TCR mobilization and clustering in response to TCR agonist coated beads was greatly enhanced in *Mgat5*$^{-/-}$ cells [36]. This was associated with enhanced signaling; notably TCR-dependent tyrosine phosphorylation, actin microfilament reorganization, and Ca^{+} mobilization were greater in *Mgat5*$^{-/-}$ T cells 1–10 min after exposure to ligand. Responses to phorbol esters, a cell-permeable stimulator of signaling downstream of TCR was normal, consistent with the defect in *Mgat5*$^{-/-}$ T cells residing at the cell surface [36].

5. Galectins interact with Mgat5 glycans to modulate TCR signalling

Mgat5-modified glycans on TCR may bind galectins and form a multivalent lattice that inhibits TCR recruitment into the immune synapse. We tested this hypothesis and observed that TCR complex is bound to galectin-3 in a Mgat5-dependent manner at the cell surface [36]. Treatment of wild type T cells with lactose or lactosamine to dissociate the galectins from their endogenous ligands resulted in enhanced TCR clustering, in this regard creating a phenocopy of *Mgat5*$^{-/-}$ [36]. Furthermore, lactose pretreatment rendered wild type T cells hypersensitive to TCR agonist as indicated by tyrosine phosphorylation of many of the usual downstream signaling proteins 1 min after antigen exposure. Lactose treatment of wild type cells differed from the *Mgat5*$^{-/-}$ phenotype in other ways, notably Ca^{+} mobilization was not stimulated by lactose plus antigen, suggesting there may be positive effects of galectins on other receptors such as CD45 which has polylactosamine on *O*-glycans (Demetriou, Dennis, unpublished). In other studies, exogenous galectin-1 has been reported to antagonize TCR clustering in lipid rafts and prevent sustained signaling [40]. Exogenous galectin-1 added to T cells also binds to the *O*-glycans on CD45 and can modulate T cell death in vitro [41].

The galectin family is conserved in metazoans, and members have either one or two carbohydrate-recognition domains (CRD). Galectins-1 and -3 have one CRD and form homodimers with CRDs spaced ~ 50 Å apart and oriented in opposing directions [42], a feature ideally suited for cross linking glycoproteins that posses multiple glycans [43]. The monomeric affinity of galectins for lactosamine and lactose are in the 10^{-3} M range [44], comparable to the affinity of peptide-MHC-induced oligomerization of TCR measured in solution [45], and therefore compatible with exchange between TCR clustering and TCR-galectin binding. The affinity of TCR for an activating peptide-MHC is greater than the galectin interaction (10^{-4}–10^{-5} M), and presumably can compete favorably with the TCR–galectin lattice for immune synapse formation. β1,6GlcNAc-branching and associated *N*-acetyl-polylactosamine enhance the affinity of glycoproteins for the galectins [44], and presumably contribute to the greater avidity of the lattice in Mgat5-expressing cells compared to the *Mgat5*$^{-/-}$ T cells. Receptors in synaptic junctions of the nervous system may also be regulated by galectin–glycoprotein interactions. In this regard, differential glycosylation of the proteoglycan agrin converts its activity to either promote or inhibit receptor aggregation in T cells, as well as postsynaptic clustering of acetylcholine receptors [46].

Naïve T cells circulating in the periphery have undergone TCR rearrangement to generate molecular diversity followed by selection in the thymus to reduce self-reactivity. Mature $CD4^{+}$ and $CD8^{+}$ T cells express a single TCR, which is dispersed over the cell surface in the resting state. TCR does not recognize antigenic peptides in solution, but does so at the surface of an antigen-presenting cell (APC) when the peptide is bound to the major histocompatibility complex (MHC). The topology of this cell–cell interaction requires reorganization of the TCR along with accessory adhesion receptors (CD4, CD8, LFA-1, ICAM-1) into a

macromolecular structure called the immune synapse [47]. TCRs operate at low affinity, binding MHC occupied by peptide that may differ in only a couple of amino acids from self-peptides, but initiation of the synapse organizes the receptors for multivalent and higher avidity binding. Intracellular signaling immediately following productive engagement of TCR induces cytoskeleton reorganization, which mobilizes receptors in the plane of the membrane and reorganizes them into an immune synapse. TCR affinity for peptide-MHC triggers receptor clustering within 30 s, and immune synapse formation matures over 60 min [47]. If the TCR-dependent intracellular signals are sustained over this time, T cells enter S phase and the antigen-specific clones rapidly expand [45]. The early stages of immune synapse formation are dynamic and allow time for TCR to sample various peptide-MHC, which is believed to minimize self-reactivity events. Importantly, the avidity created by receptor clustering in the immune synapse allows T cells to response to relatively low-affinity foreign peptides [48]. In this regard, Th1 $CD4^+$ helper cells (i.e. produce INF-γ and IL-2) form immune synapses but none are observed in Th2 $CD4^+$ cells (IL-10, IL-4). Consistent with the signal amplifying function of synapses, Th1 cells are more sensitive to low-affinity peptides than Th2 cells [49]. As might be expected with the enhanced synapse formation observed in *Mgat5*$^{-/-}$ T cells, a proportionate increase in Th1 cytokine production is observed following TCR stimulation (Dennis, Li, Pawling, in preparation).

Recycling of glycoproteins through the endocytic pathway and back to the cell surface does not appear to involve remodeling of *N*-glycan branching [50]. Therefore, changes in levels of *N*-glycan branching at the cell surface occur through de novo synthesis of glycoproteins. The time scale of this process is hours, whereas TCR-dependent tyrosine phosphorylation, Ca^+ mobilization and actin rearrangement occurs in minutes after agonist-induced receptor clustering. As such, Mgat5-dependent regulation of TCR sensitivity in resting cells is a long time-scale or slow form of negative feedback. Ras-Raf-Ets, which is downstream of TCR, is a positive regulator of Mgat5 gene expression [16,17]. Therefore, the steady-state activity of Ras-Raf-Ets signaling in resting T cells presumably regulates Mgat5 activity and thereby TCR threshold to antigen. This type of delayed negative feedback is observed in many pathways, and provides a mechanism to fine-tune receptor sensitivity to match ambient conditions [51]. It is possible that Mgat5 expression and TCR sensitivity on circulating naïve T cells depend in part on the tissue environments.

Following activation, T cells become more sensitive to antigen stimulation, even though TCR levels and the intrinsic affinity of TCR for peptide-MHC does not change [52,53]. Several mechanisms have been proposed including enhanced TCR dimerization occurring selectively in lipid rafts [52]. Additionally, receptor-associated kinase lck increases, and signaling sensitivity downstream of TCR is enhanced in activated cells [53]. *Mgat5* transcription, enzyme activity and glycan products are increased following activation [36,54], and this might be expected to dampen TCR receptor clustering and decrease sensitivity. However, the influence of Mgat5-modified glycans may shift from that of the TCR to integrins following activation. In support of this idea, Mgat5-modified glycans in substratum attached cells promote focal adhesion turnover and cell motility. Leukocyte migration into the peritoneal cavity in response to an injection of thioglycolate was delayed in *Mgat5*$^{-/-}$ mice, and the cells were more adhesive on fibronectin (Cheung, Granovsky, Dennis, in preparation). Adhesion of activated T cell on fibronectin was also greater for *Mgat5*$^{-/-}$ compared to *Mgat5*$^{+/+}$ cells (Demetriou, Granovsky, unpublished). These observations suggest that Mgat5-modified glycans may stimulate T cell motility and associated signaling via PI3 kinase/PKB pathway, thereby sustain activation and survival of the T cells.

6. Delayed cancer progression in Mgat5-deficient mice

Transgenic mice expressing oncogenes under the control of tissue-specific promoters more closely reflect the natural history of cancer, than does injection of cultured tumor cell lines into mice. Therefore, transgenic mice expressing polyoma virus middle T (PyMT) oncoprotein under the control of the murine mammary tumor virus (MMTV) promoter were intercrossed with Mgat5-deficient mice [32]. The PyMT transgenic mice develop multifocal mammary carcinomas that progress and metastasize to the lungs. PyMT is a potent oncoprotein with multiple binding domains for docking of signaling proteins that stimulate proliferation. Tyrosine at position Y315/Y322 is phosphorylated by pp60c-src to create a binding site for p85, a positive regulator of PI3 kinase and the downstream kinases PDK1 and PKB [55]. An NPTY motif in PyMT is also phosphorylated, creating a binding site for the PTB domain of Shc protein, and downstream activation of the Ras pathway [55–57]. Polyoma virus transformation induces Mgat5 activity and *N*-glycan products [19]. Importantly, the PyMT transgenic cancer model activates intracellular signaling pathways known to be causal in human cancers.

In PyMT *Mgat5*$^{-/-}$ mice on a 129 × FVB background, mammary tumors were delayed and grew more slowly than those in heterozygous and wild type mice [32]. All *Mgat5* genotypes showed multifocal tumor initiation, but the fraction of proliferating cells was significantly lower (~ 10%) in PyMT *Mgat5*$^{-/-}$ carcinomas compared to that of PyMT *Mgat5*$^{+/-}$ mice. With exponential expansion of cells in the tumor, the impact of this rate difference was reflected in a ~ 5-fold reduction in tumor burden by 28 weeks. Lung metastases were reduced 20-fold in PyMT *Mgat5*$^{-/-}$ mice compared to wild type and heterozygous mice. Strain-dependent modifier genes appear to influence the Mgat5–PyMT interaction. *Mgat5*$^{-/-}$ mutation markedly sup-

pressed PyMT tumor growth on FVB background, but on the 129 background, tumors grew more slowly and the effect of the Mgat5-deficiency was less pronounced (Pawling, Dennis, in preparation).

PyMT *Mgat5*$^{-/-}$ tumors were deficient in PKB/Akt activation while another extracellular responsive kinase, Erk, was similar in both PyMT *Mgat5*$^{-/-}$ and PyMT *Mgat5*$^{+/-}$ tumors [32]. PKB/Akt activation is associated with cell motility [58,59], and indeed *Mgat5*$^{-/-}$ tumor cells were deficient in membrane ruffling and actin filament turnover. This suggests that Mgat5-modified glycans collaborate with PyMT to activate PI3 kinase and PKB for optimal tumor growth and metastasis. Interestingly, PyMT-dependent tumor growth is dosage sensitive to several intracellular signaling proteins. Notably, delays in tumor growth have been observed in PyMT transgenic mice on a *Grb2*$^{+/-}$ genetic background, an adapter protein in the Ras pathway [60], and also on an *Ets-2*$^{+/-}$ background, a transcription factor downstream of Ras [61].

A minority (~ 5%) of the PyMT *Mgat5*$^{-/-}$ tumors acquired a fast-growth phenotype, apparently escaping *Mgat5*$^{-/-}$ dependent growth suppression [32]. Mgat5-modified glycans were not reexpressed in the fast-growth PyMT *Mgat5*$^{-/-}$ tumors. Presumably, rare mutations in genes that can relieve *Mgat5*$^{-/-}$ growth suppression occur, and the acquired growth advantage produces fast-growing tumors. Consistent with this interpretation, we observed restoration of Akt/PKB activation and membrane ruffling [32]. In a similar manner, tumors in transgenic mice with a mutant PyMT transgene that lacks the P85/PI3 kinase-binding site undergo secondary compensating events, such as amplification of ErbB2 and c-ErbB3 receptors [62]. Our results suggest that dependence on Mgat5 for rapid tumor growth can be overcome possibly by additional mutations that amplify PI3 kinase/PKB signaling, such as loss of PTEN function. PTEN is a D3-phosphoinositides phosphastase that counteracts PI3 kinase and is commonly mutated in human cancer [63].

In cancer cells, PI3K products are specifically elevated relative to other phosphoinositides, and serve as positive regulators of the membrane remodeling required for cell motility, proliferation, survival and endosomal trafficking [64]. The phenotype of Mgat5-deficient cells is consistent with suppression of PI3K activation, notably slow growth, decreased phosphorylation of PKB, decreased motility and focal adhesion signaling. However, the glycoproteins bearing Mgat5-modified glycans that mediate this phenotype remain to be identified. With our observations that Mgat5-modified glycans regulate TCR clustering and internalization, it is possible that Mgat5 glycans regulate multiple receptors in a similar manner. As a working hypothesis, Mgat5-modified glycans may alter the distribution, trafficking and threshold for ligand-dependent signaling by certain receptors upstream of PI3K signaling. Integrin-mediated cell motility is dependent on PI3K and PKB activation [58,59], and motility is markedly enhanced by Mgat5 gene expression in tumor cells [22] (Partridge, Dennis, in preparation). The α_5 and β_1 chains of fibronectin receptor have 14 and 12 possible *N*-glycosylation sites, respectively, a portion of which contain Mgat5-modified glycans [65,66]. Overexpression of Mgat5 in MvLu1 epithelial cells enhances β1,6 GlcNAc-branching on α5β1 integrin, and enhances cell motility and growth [22]. Integrin endocytosis is stimulated by exogenous galectin-3 in mammary cancer cells, and this can be blocked by addition of lactose [67], suggesting a dependence on a binding partner. Recombinant galectin-3 and galectin-8 also promotes cell spreading [67] and integrin-dependent signaling [68], suggesting a role of the glycans in both integrin turnover rates and signaling.

The cytosolic tail of β integrins contains tyrosine-phosphorylation motifs YXXϕ where X is any amino acid and ϕ is a bulk hydrophobic residue [69]. The YXXϕ sequence binds clathrin adapter proteins AP-1/AP-2, and is required for endosomal recycling of receptors back to the leading edge of the cell. The kinetics of integrin recycling affects the magnitude and duration of signaling, as suggested by studies examining the distribution of GFP fused to the cytoplasmic tail of β1 integrin [70]. In stationary cells, integrin–GFP was highly motile, marking adhesion foci that moved short distances toward the cell center and rapidly turned over. In migrating cells, the focal adhesion formed as membrane lamenapodia protrude forward but the integrin–GFP marked complexes were larger and more persistent. It is possible that binding of galectins to Mgat5-modified glycans on integrins stabilizes focal adhesions or slows their endocytosis and thereby increases signaling.

Integrin-mediated adhesion and motility stimulate the downstream pathways common to growth factor receptors, and may do so by interacting with receptor tyrosine kinases, notably PDGF and EGF receptors [71]. Cytokine-dependent stimulation of these receptor tyrosine kinases by cognate ligands induces autophosphorylation, creating SH2 binding sites for the regulatory protein P85, which then recruits the catalytic subunit of PI3K [72,73]. The PI3K produces PIP3,4 and PIP3,4,5 tether PH-domain containing proteins to the inner surface of the membrane, including PKB, PDK1, Tec kinases, plexstrin, PLCγ, and guanine nucleotide exchange factors. The recruitment and activation of these proteins stimulate cytoskeleton remodeling that drives cell motility, cell proliferation and survival in tumor cells (reviewed in Ref. [74]). Recent studies show that integrins associate with EGF receptors, and integrin-mediated adhesion stimulates receptor phosphorylation. The integrin cytoplasmic tail, recruitment of c-Src, and the adaptor protein p130Cas are required for EGF receptor activation in response to integrin-mediated adhesion [75]. These studies suggest a mechanism for positive feedback between cytokine receptor and integrin-mediated signaling, which should amplify PI3K/PKB activation and downstream phenotypes.

The steady state levels of PKB phosphoproteins is lower in Mgat5-deficient cells indicating that steady state signaling through the PI3K/PKB pathway is suppressed [32,36]. PI3K products also regulate vesicular endocytosis, and the kinetics of receptor trafficking can markedly affect signaling by EGF and c-Met receptors [76]. Cytokine–receptor complexes signal from the cell surface and early endosome prior to their sorting into the late endosomes for either destruction or recycling [77]. Preventing EGF receptor endocytic destruction shunts receptors to the recycling pathway, and promotes signaling and cell transformation [78]. We are currently extending our work with TCR to determine whether galectin–glycoprotein interactions also regulate the residency-time of other Mgat5-modified receptors on the plasma membrane, or in early endosomes, and thereby the threshold to ligands.

7. Threshold effects of glycosylation in systems of exponential cell proliferation

Individual cells typically show small variances for many molecular parameters, such as density of cell surface receptors, and these together confer a Gaussian spectrum of responsiveness in the cell population. In addition, the protein glycosylation increases molecular heterogeneity further, and presumably the functional diversity within cell populations (Fig. 1). Plasticity or variability of responsiveness in the T lymphocyte population can enhance the range of possible systemic responses to pathogens [79,80]. Maximum fitness of immune system balances sensitivity to many foreign antigens with the avoidance of self-reactivity. An immune system optimized for highly synchronous T cell responses might be very effective against some pathogens, but at the expense of a limited T cell repertoire. As a result, precise molecular recognition by the immune response may be rewarded less in evolutionary terms than lower affinity but increased molecular diversity providing more opportunities to hit the target [81]. Therefore, glycoform variations even among clones that have the same rearranged TCR gene should result in heterogeneity among responding cells, as our studies on the Mgat5-deficient mice have demonstrate [36]. Less synchrony allows time for negative feedback to limit immunopathology. As depicted in Fig. 1, glycan heterogeneity results in a glycoform distribution in each T cell (K_i), which increases the variance within the cell population (Z_i) [80]. We have experimentally altered the glycoform distribution in Mgat5-deficient mice, which reduces glycan structural heterogeneity, and shifts the T cell population to a more hypersensitive state. This increases the probability of autoimmune T cell activation in *Mgat5*$^{-/-}$ mice. Once triggered, the autoimmune T cell clone(s) expand exponentially causing systemic disease [79] (Fig. 2A).

Cancer progression is characterized by increasing heterogeneity over time for many phenotypes. In addition to the normal glycoform diversity, tumor cells display a wide

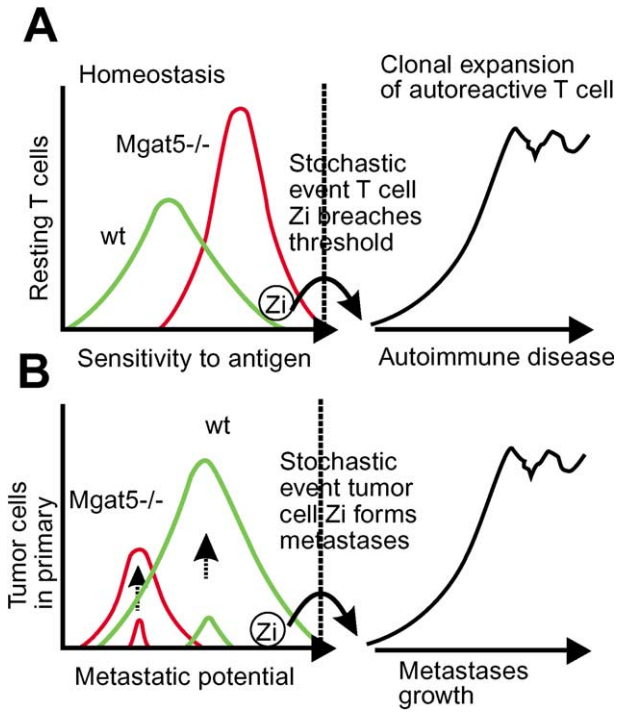

Fig. 2. (A) The frequency of T cells as a function of antigen sensitivity in wild type and *Mgat5*$^{-/-}$ mice. The onset of autoimmune disease occurs when one or a few autoreactive T cells are activated and rapidly expand in numbers [79]. This event is more probable in *Mgat5*$^{-/-}$ mice which have a lower TCR threshold for activation, and once the threshold is crossed by an autoreactive T cell (Z_i), this clone expands rapidly causing pathology. (B) Growth rates of the primary tumor are lower in Mgat5 mutant mice (red) than in wild type mice (green) as indicated by the vertical arrows. Mgat5 also promotes the metastatic phenotype as indicated by leftward placement of the *Mgat5*$^{-/-}$ population. At a low frequency, tumor cells escape the primary, establish colonies in secondary organs and expand to form secondary tumors. Genomic instability combined with exponential growth increases phenotypic heterogeneity in primary tumors, thereby generating cells with metastatic potential.

range of altered glycosylation compared to nontransformed cell counterparts [82], and Mgat5 is a major contributor to *N*-glycan heterogeneity. Progressive tumor cell growth from 1 cell to $>10^{10}$ coupled with genetic instability increases the probability of generating cells capable of establishing metastases in vital organs. Mgat5 glycans on tumor cells contribute to PI3 kinase/PKB activation, a critical pathway for tumor cell growth, motility and resistance to apoptosis. Based on our studies, Mgat5 glycans contribute directly to focal adhesion turnover and invasion, but in addition, their positive effects on tumor growth may speed the generation of metastatic cells (Fig. 2B). We suggest that highly cooperative events such as TCR and integrin receptor signaling at the molecular level are sensitive to changes in Mgat5-dependent *N*-glycosylation. Cellular interactions that are sensitive to glycoform variation include autoimmunity and cancer metastasis. The triggering of rare self-reactive T cells in autoimmune patients, and the growth of metastatic colonies in cancer patients are both highly selective events over a large population of cells. In these diseases, small

functional differences in initiation thresholds for pathology have the potential to be magnified greatly by subsequent clonal expansion of the offending cells (Fig. 2B).

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