Ameliorating Skin-Homing Receptors on Malignant T Cells with a Fluorosugar Analog of *N*-acetylglucosamine: P-Selectin Ligand Is a More Sensitive Target than E-Selectin Ligand

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Expression of E- and P-selectin ligands is required for T cell entry into skin. Sialyl Lewis X moieties are critical for ligand activity and are elevated on malignant skin-homing T cells. We hypothesize that these glycosylations are selectable targets for treating the dermal tropism associated with cutaneous lymphomas. In this study, we analyzed the efficacy of a novel 4-fluorinated analog of *N*-acetylglucosamine (GlcNAc) on E- and P-selectin ligands expressed by malignant skin-homing T cells. We also examined the specificity of 4-F-GlcNAc (2-acetamido-1,3,6-tri-*O*-acetyl-4-deoxy-4-fluoro-D-glucopyranose) action by contrasting the effects on sialyl Lewis X expression displayed by P-selectin glycoprotein ligand-1 (PSGL-1) with sialylated *O*-glycans expressed by CD43. Using parallel-plate flow analysis, we found that 4-F-GlcNAc elicited 5-fold more potent inhibition on P-selectin ligand activity than on E-selectin ligand activity. To determine whether glycosylations conferring E- and P-selectin ligand activities were inhibited, we analyzed the expression of sialyl Lewis X and sialyl-fucosylated core 2 *O*-glycan (CHO-131 antigen), respectively. We found that 4-F-GlcNAc treatment resulted in dose-dependent ablation of sialyl Lewis X and CHO-131 antigen expression on PSGL-1, whereas sialylated *O*-glycans on CD43 were minimally affected. These results indicate that 4-F-GlcNAc treatment can selectively downregulate the P-selectin ligand activity and potentially prevent dermal dissemination of cutaneous lymphomas.

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INTRODUCTION

Sialyl Lewis X moieties, defined by α2,3 sialylated and α1,3 fucosylated *N*-acetyllactosamine, are characteristically expressed on skin-homing T cells and are necessary for T cell trafficking to skin (Picker *et al.*, 1990; Berg *et al.*, 1991). Detection of this moiety with the anti-sialyl Lewis X mAb HECA-452 confers a skin-tropic phenotype of T cells and is characteristically displayed on P-selectin glycoprotein ligand-1 (PSGL-1), otherwise known as cutaneous lymphocyte-associated antigen (CLA) (Fuhlbrigge *et al.*, 1997). Expression of HECA-452 antigen and another sialyl Lewis X epitope recognized by newly described mAb, CHO-131, directly correlates with the capacity of PSGL-1 to interact with dermal endothelial E- and P-selectin, respectively (Fuhlbrigge *et al.*, 2002; Walcheck *et al.*, 2002). These Ca^{2+}-dependent, vascular endothelial receptors mediate tethering and rolling of T cells on dermal microvessel surfaces, initiating recruitment into skin for the maintenance of immune surveillance or pathologic inflammatory processes (Davis and Smoller, 1992; Rossiter *et al.*, 1994; Robert and Kupper, 1999). Notably, CLA is conspicuously upregulated on malignant T cells in patients with cutaneous T cell lymphoma (CTCL) and directly correlates with the unique pattern of skin involvement, providing the opportunity to target these sialyl Lewis X moieties and attenuate the dermal dissemination of malignant T cells (Borowitz *et al.*, 1993; Robert and Kupper, 1999; Ferenczi *et al.*, 2002; Yamaguchi *et al.*, 2003).

Mice deficient in endothelial E- and P-selectin (Staite *et al.*, 1996; Catalina *et al.*, 1999; Smithson *et al.*, 2001; Hirata *et al.*, 2002) or in α1,3 fucosyltransferase VII (Smithson *et al.*, 2001; Erdmann *et al.*, 2002), which fucosylates PSGL-1, exhibit defective cutaneous inflammatory responses, providing pharmacologic rationale for investigating strategies to prevent dermal dissemination of malignant T cells. We

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Abbreviations: BAG, benzyl-O-N-acetylgalactosamide; CLA, cutaneous lymphocyte-associated antigen; CTCL, cutaneous T-cell lymphoma; 4-F-GlcNAc, 2-acetamido-1,3,6-tri-O-acetyl-4-deoxy-4-fluoro-D-glucopyranose; GlcNAc, N-acetylglucosamine; PBS, phosphate-buffered saline; PSGL-1, P-selectin glycoprotein ligand-1

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have previously shown that a novel *N*-acetylglucosamine (GlcNAc) sugar substituted with a fluorine atom at the carbon-4 position, named 4-F-GlcNAc (2-acetamido-1,3,6-tri-*O*-acetyl-4-deoxy-4-fluoro-D-glucopyranose), can block the synthesis of *N*-acetyllactosamine structures, thereby inhibiting sialyl Lewis X expression and selectin ligand activity (Woynarowska *et al.*, 1996). In particular, 4-F-GlcNAc downregulates CLA expression on *ex vivo*-cultured human CLA$^+$ T cells by incorporating into PSGL-1 *O*-glycans and preventing the synthesis of sialyl Lewis X (Dimitroff *et al.*, 2003a). Using an *in vivo* model of allergic contact dermatitis that is dependent on effector T cell E- and P-selectin ligand expression, we demonstrate that 4-GlcNAc prevents T cell E-selectin ligand expression and T cell-mediated inflammation (Dimitroff *et al.*, 2003b). Whereas other carbohydrate-dependent processes required for antigen-dependent inflammation, such as naïve lymphocyte homing to lymph nodes and dendritic cell migration into lymph nodes, do not appear to be affected by 4-F-GlcNAc (Dimitroff *et al.*, 2003b). These effects on CLA expression and function have heightened our enthusiasm for potentially controlling the pathogenesis of cutaneous lymphomas.

In this report, we examined the efficacy and potency of 4-F-GlcNAc on E- and P-selectin ligand activity displayed by malignant human CLA$^+$ Hut 78 T cells derived from a patient with CTCL. We also investigated the modulation of sialyl Lewis X epitopes, HECA-452 antigen, and CHO-131 antigen, which directly correspond to E- and P-selectin ligand activity on PSGL-1. The specificity of 4-F-GlcNAc anticarbohydrate action was also explored by elucidating 4-F-GlcNAc efficacy on sialylated *O*-glycans expressed by CD43. Compared with HECA-452 antigen and E-selectin ligand inhibition, CHO-131 antigen and P-selectin ligand activity of PSGL-1 were 5-fold more sensitive to 4-F-GlcNAc treatment. Of note, sialyl Lewis X epitopes and E-selectin ligand activity on glycolipid scaffolds, which represents $\sim$50% of total cellular activity, were unaffected by 4-F-GlcNAc treatment, and 4-F-GlcNAc efficacy on CD43 sialylated *O*-glycans was insignificant. Mechanistically, formation of the *N*-acetyllactosamine corresponding to CHO-131 antigen and P-selectin ligand at the N-terminus of PSGL-1 was particularly sensitive to 4-F-GlcNAc treatment. These findings indicate that E- and P-selectin-binding determinants could be opportune targets for controlling the dermal dissemination of malignant skin-homing T cells.

RESULTS

We have previously shown that 4-F-GlcNAc can modulate E- and P- selectin ligand activities on *ex vivo* cultures of normal CLA$^+$ T cells (Dimitroff *et al.*, 2003a). Since E- and P-selectin ligand activities on T cells confer a skin-tropic phenotype, we believe that these ligand activities are targetable entities for controlling the dermal tropism associated with T cell-mediated inflammation and cancer. With regard to CTCL, there is strong evidence indicating that CLA is upregulated on malignant T cells and is associated with cutaneous involvement and progression of CTCL (Borowitz *et al.*, 1993; Heald *et al.*, 1993; Robert and Kupper, 1999; Ferenczi *et al.*, 2002). As T cell E-selectin ligand activity is directly related with CLA expression (Fuhlbrigge *et al.*, 1997) and a subset of CLA$^+$ T cells bind P-selectin (Walcheck *et al.*, 2002; and Ni Z, Campbell JJ, G. Niehans G, Walcheck B. CHO-131 reactivity with human T cells corresponds with a high P-selectin binding activity. Manuscript submitted for publication), we hypothesize that the association between elevated CLA expression and CTCL progression is owing to concomitant elevations in P- and E-selectin ligand activities on malignant T cells.

In the following analyses, we determined the sensitivity and specificity of 4-F-GlcNAc efficacy on E- and P-selectin carbohydrate recognition determinants relative to effects on other glycoconjugates expressed by malignant human T cells. We utilized Hut 78 cells, which are malignant CD4$^+$ T cells derived from a patient with Sezary syndrome, as a malignant T cell model for biochemical and antiadhesion analysis of 4-F-GlcNAc efficacy (Gazdar *et al.*, 1980). Before incubations in 4-F-GlcNAc and other control glycosylation inhibitors, we assessed treatments on Hut 78 cultures in log phase growth to determine pertinent concentrations for analysis of glycan and adhesion modulation. We found that 4-F-GlcNAc concentrations less than 0.15 mM ($<IC_{10}$) did not affect T cell growth and did not affect the expression of T cell marker CD4 and of PSGL-1 (data not shown). Benzyl-*O*-*N*-acetylgalactosamide (BAG), a metabolic inhibitor of *O*-glycan biosynthesis, was tested at concentrations previously shown to inhibit sialyl Lewis X and/or *O*-glycan expression and did not affect cell growth and protein synthesis (data not shown) (Dimitroff *et al.*, 2003a).

4-F-GlcNAc efficacy on E- and P-selectin ligand expression on malignant T cells

Using the parallel-plate flow chamber under physiologic shear forces, we examined the capacity of Hut 78 cells treated with 4-F-GlcNAc to bind E- and P-selectin. As expected, control neuraminidase treatment completely abrogated both E- and P-selectin ligand activities on Hut 78 cells (Figure 1). However, while E-selectin ligand activity was significantly diminished in a dose-dependent manner from 10 to 100 μM 4-F-GlcNAc, only a maximal 50% inhibition of untreated control was observed at the highest dose (statistically significant difference compared with untreated control; $P<0.001$) (Figure 1). Likewise, protease (bromelain) treatment and BAG treatments exhibited a maximal reduction of E-selectin ligand activity by 50% (Figure 1). These results suggested that a residual E-selectin ligand activity was mediated by sialylated glycolipid and that 4-F-GlcNAc only affects E-selectin-binding determinants on glycoprotein. On the contrary, we found that 4-F-GlcNAc caused a more potent dose-dependent reduction in P-selectin ligand activity, in which the lowest dose of 10 μM resulted in a significant reduction by 44% and the highest dose at 100 μM eliminated activity (statistically significant difference compared with untreated control) (Figure 1). Removal of *O*-glycans and membrane protein by BAG (2 mM) and bromelain treatments, respectively, similarly eliminated P-selectin ligand activity (Figure 1), confirming PSGL-1's role as the principal T cell P-selectin ligand (Vachino *et al.*, 1995).

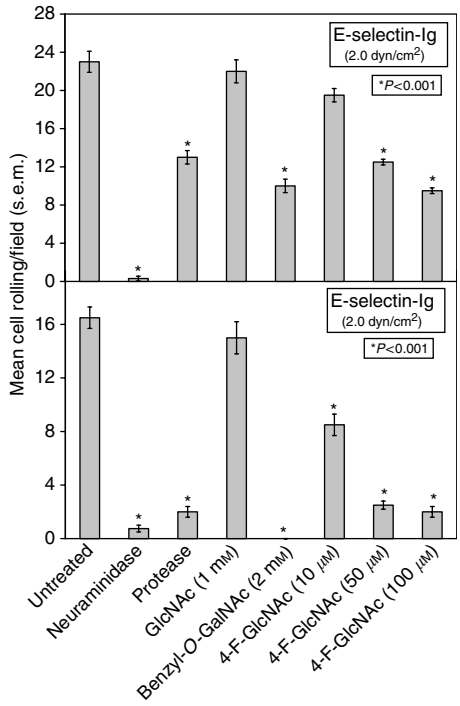

Figure 1. Parallel-plate flow analysis of 4-F-GlcNAc efficacy on malignant T cell E- and P-selectin ligand activities. At 2.0 Dyn/cm^2, we analyzed the rolling activity of Hut 78 cells treated with neuraminidase, protease (bromelain), 1 mM GlcNAc, 2 mM BAG, or 10, 50 or 100 μM 4-F-GlcNAc. Mean cell rolling (SEM) frequencies on immobilized human E- or P-selectin chimera were quantified from a minimum of three fields, and experiments were repeated a minimum of three times. Cell rolling on human IgG or in assays performed in the presence of 5 mM EDTA was not evident. (*$P<0.001$; statistically significant difference compared with untreated control, Student's paired *t*-test).

Complementary experiments were conducted using flow cytometry with mAbs HECA-452 and CHO-131 to determine whether sialyl Lewis X (HECA-452 antigen) and/or sialyl-fucosylated core 2 *O*-glycans (CHO-131 antigen) were downregulated following 4-F-GlcNAc treatment. Expression of HECA-452 and CHO-131 antigen confers the presence of carbohydrate recognition determinants on T cells for binding to E- and P-selectin, respectively (Walcheck *et al.*, 2002). Using Hut 78 cells treated identically to cells used in cell rolling experiments, we found that neuraminidase treatment caused marked reductions in both HECA-452 and CHO-131 antigen levels, whereas control phosphate-buffered saline (PBS)-treated cells and control GlcNAc-treated cells showed no change in antigen levels (Figure 2a and b). We found that 4-F-GlcNAc inhibited both HECA-452 and CHO-131 antigen expression, but as observed in cell-binding experiments, 10 μM 4-F-GlcNAc resulted in a 35% reduction in CHO-131 antigen expression, while having no effect on HECA-452 antigen at 10 μM (Figure 2a and b). Interestingly, HECA-452 antigen levels were not completely removed at the highest concentrations of BAG and 4-F-GlcNAc, and protease bromelain treatment only halved HECA-452 antigen expression (Figure 2a). On the contrary, 100 μM 4-F-GlcNAc, 2 mM BAG, and protease showed markedly lower levels of CHO-131 antigen compared with PBS diluent controls (Figure 2b). With the exception of protease treatment, expression of PSGL-1 polypeptide was not lowered after neuraminidase, GlcNAc, BAG, or 4-F-GlcNAc treatments, suggesting that expression of candidate selectin glycoprotein scaffolds were not affected (Figure 2c). These data corroborated with cell rolling data and showed that CHO-131 antigen was likely presented by PSGL-1 and directly correlated with P-selectin ligand activity and that HECA-452 antigen was expressed on both glycoprotein and glycolipid and directly correlated with E-selectin ligand activity.

4-F-GlcNAc efficacy on sialyl Lewis X and sialyl-fucosylated core 2 *O*-glycans displayed by PSGL-1

To investigate 4-F-GlcNAc efficacy on the expression of sialyl Lewis X and sialyl-fucosylated core 2 *O*-glycans displayed on glycoprotein(s), we immunoblotted lysates from 4-F-GlcNAc-treated Hut 78 cells with mAb HECA-452 or mAb CHO-131, respectively. MAb HECA-452- and CHO-131-reactive glycoprotein(s), principally detected at 120 and 240 kDa in lysates from untreated (PBS) Hut 78 cells, was completely eliminated following neuraminidase or BAG treatments (Figure 3a). Furthermore, treatment with 4-F-GlcNAc elicited a dose-dependent decrement in HECA-452 and CHO-131 antigen on glycoprotein with complete disappearance at 100 μM, a 20-fold lower concentration than BAG (Figure 3a and b). Moreover, densitometric analysis of CHO-131 staining of glycoprotein from cells treated with 10 μM 4-F-GlcNAc revealed a significant diminution compared with PBS control ($P<0.01$; Student's paired *t*-test) (Figure 3b). Note that molecular control, GlcNAc, had no effect on antigen expression and that equivalent levels of β-actin were detected in immunoblots from lysates displaying low to nil levels of HECA-452 and CHO-131 antigen, suggesting that reductions in sialyl Lewis X and in sialyl-fucosylated core 2 *O*-glycans were independent of protein synthesis (Figure 3a and b).

To examine directly the effects of 4-F-GlcNAc on candidate E- and P-selectin glycoprotein ligand at 120 and 240 kDa, we immunoprecipitated PSGL-1 with mAb KPL-1 and blotted with mAb HECA-452, mAb CHO-131, E-selectin-Ig chimera, or mAb KPL-1. As PSGL-1 characteristically resolves at ~120 and 240 kDa and is a major glycoprotein on T cells that displays HECA-452 antigen and E-selectin-binding determinants (Borges *et al.*, 1997; Fuhlbrigge *et al.*, 1997, 2002; Dimitroff *et al.*, 2003a), we presumed that PSGL-1 was the major glycoprotein bearing E- and P-selectin carbohydrate recognition determinants on Hut 78 cells. As shown in the anti-PSGL-1 immunoblot, PSGL-1 was present at comparable levels in immunoprecipitates from all treated cells and isotype Ab control immunoprecipitates did not contain any PSGL-1 (Figure 4a). Compared with staining of anti-PSGL-1 immunoprecipitates from PBS- or GlcNAc-treated cells, neuraminidase and BAG treatments significantly reduced HECA-452 and CHO-131 antigen and E-selectin-binding determinants on PSGL-1 ($P<0.01$; Student's paired *t*-test) (Figure 4a and b). 4-F-GlcNAc treatments resulted in a dose-dependent reduction in HECA-452 and CHO-131 antigen and E-selectin ligand on PSGL-1 (Figure 4a and b).

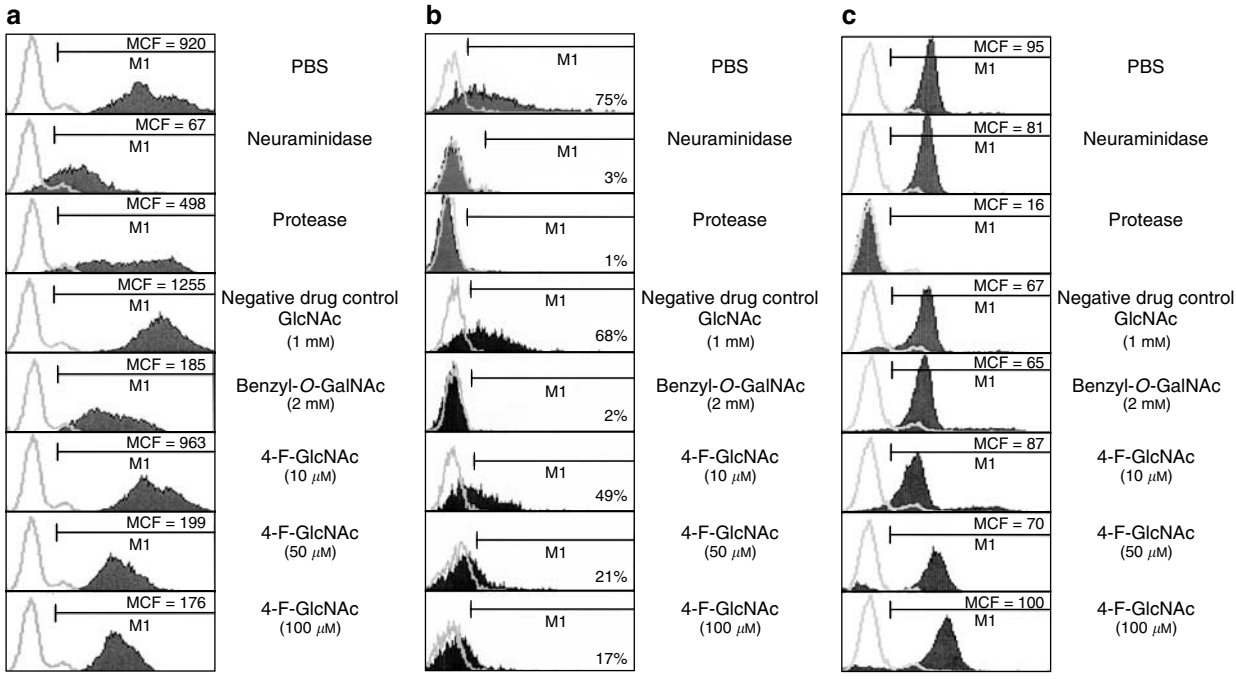

Figure 2. Flow cytometric analysis of HECA-452 and CHO-131 antigen and of PSGL-1 expression on malignant T cells treated with 4-F-GlcNAc. Hut 78 cells treated with neuraminidase, protease (bromelain), 1 mM GlcNAc, 2 mM BAG, or 10, 50, or 100 μM 4-F-GlcNAc were stained with (**a**) mAb HECA-452, (**b**) mAb CHO-131, or (**c**) anti-PSGL-1 mAb PL-2 and respective fluorochrome-conjugated secondary Ab. Data in (**a**) and (**c**) are presented as MCF of positive stained cells, whereas data in (**b**) are presented as % positive stained cells. Experiments were repeated a minimum of three times. MCF = mean channel fluorescence.

Notably, 4-F-GlcNAc markedly inhibited CHO-131 antigen, which directly corresponds to P-selectin ligand activity, on PSGL-1 at 10 μM, whereas 10 μM 4-F-GlcNAc did not greatly affect HECA-454 antigen and E-selectin ligand levels on PSGL-1 (Figure 4a and b). As CHO-131 antigen directly corresponds to P-selectin ligand expression (Walcheck *et al.*, 2002), P-selectin ligand activity appeared to be more sensitive to 4-F-GlcNAc treatment than E-selectin-binding determinants on PSGL-1.

To investigate the selectivity of 4-F-GlcNAc efficacy on endothelial selectin recognition determinants, we analyzed the expression of sialylated *O*-glycans on T cell sialomucin, CD43, following 4-F-GlcNAc treatment. CD43 characteristically expresses high levels of sialylated *O*-glycans (Piller *et al.*, 1988; Mukasa *et al.*, 1999), serving as an analogous counterpart to PSGL-1 for the assessment of 4-F-GlcNAc's effects on glycosylations expressed by an *O*-glycosylated glycoprotein in the same cell. We immunoblotted lysates from treated Hut 78 cells with mAb L60, which recognizes sialylated *O*-glycan epitopes on CD43 (Ardman *et al.*, 1992; Dimitroff *et al.*, 2003a). As expected, neuraminidase and BAG treatments completely eliminated mAb L60 reactivity of CD43 (Figure 5a). However, 4-F-GlcNAc, even at 100 μM, did not significantly lower sialylated *O*-glycans on CD43 ((Figure 5a and b). The resistance of CD43 to 4-F-GlcNAc modulation prompted further scrutiny on the type of *O*-glycans displayed by CD43. To determine whether CD43 expresses sialyl-fucosylated core 2 *O*-glycans, we immunoprecipitated CD43 with anti-CD43 mAb 1G10 from Hut 78 cell lysates and then blotted with mAb CHO-131. Immunoblotting of anti-CD43 1G10 immunoprecipitates with mAbs 1G10 and L60 was performed in parallel to control for presence of sialo-*O*-glycosylated CD43 in anti-CD43 mAb 1G10 immunoprecipitates. Anti-CD43 immunoprecipitation of neuraminidase-treated cell lysates blotted with mAb L60 was also conducted to validate sialic acid-dependent reactivity of mAb L60. We found that while CD43 was detected in anti-CD43 immunoprecipitates blotted with mAb 1G10 and mAb L60, CHO-131 antigen was not present on CD43.

DISCUSSION

E- and P-selectin-binding determinants have been implicated as potential targets in the treatment of T cell-mediated cutaneous inflammation. We have shown that a fluorinated sugar analog of *N*-acetylglucosamine, otherwise known as 4-F-GlcNAc, functions as a potent metabolic inhibitor of *N*-acetyllactosamine and sialyl Lewis X biosynthesis. After passive cellular uptake and conversion to UDP-4-F-GlcNAc, the fluorine at carbon-4 of 4-F-GlcNAc appears to block subsequent addition of nucleotide sugar, UDP-Gal (Bernacki *et al.*, 1977; Sharma *et al.*, 1990; Woynarowska *et al.*, 1996; Dimitroff *et al.*, 2003a). Prior studies provide evidence that this mechanism of action results in the inhibition of E- and P-selectin ligand biosynthesis on normal human CLA^+ T cells and on effector lymphocytes involved in antigen-dependent cutaneous inflammation (Dimitroff *et al.*, 2003a, b). Specifically, E-selectin-binding determinants on PSGL-1 (i.e. CLA) are downregulated on *ex vivo*-cultured human CLA^+ T cells

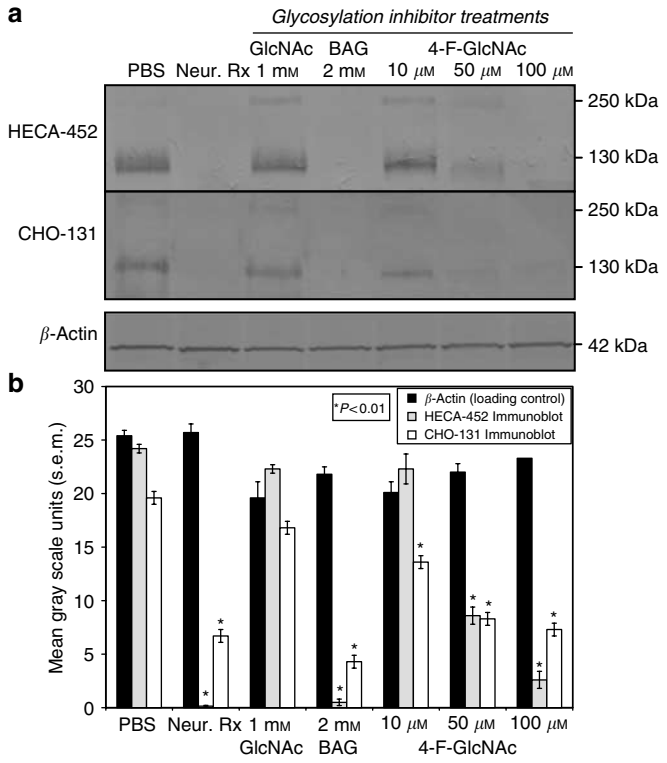

Figure 3. Western blot analysis. HECA-452 and CHO-131 antigen expression on glycoprotein expressed by malignant T cells treated with 4-F-GlcNAc. In (**a**), lysates (20 μg/lane) from Hut 78 cells treated with diluent control, neuraminidase, 1 mM GlcNAc, 2 mM BAG, or 10, 50, or 100 μM 4-F-GlcNAc were resolved on reducing 4–20% SDS-PAGE gradient gels and immunoblotted with mAbs HECA-452, CHO-131, or anti-β-actin. In (**b**), densitometric analysis of HECA-452 and CHO-131 immunostained protein from 250 to 100 kDa and of anti-β-actin-stained protein at 42 kDa was performed using NIH ImageJ software. Mean gray scale units were normalized to background staining intensities and averaged from a minimum of three experiments. (*$P<0.01$; statistically significant difference compared with PBS control, Student's paired *t*-test).

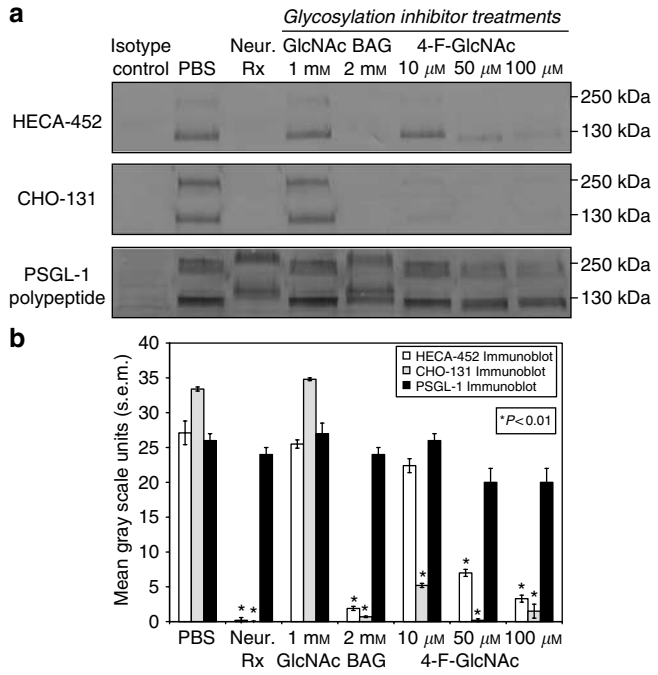

Figure 4. Western blot analysis. HECA-452, CHO-131, and E-selectin ligand expression on PSGL-1 immunoprecipitated from malignant T cells treated with 4-F-GlcNAc. In (**a**), lysates (100 μg) from Hut 78 cells treated with diluent control, neuraminidase, 1 mM GlcNAc, 2 mM BAG, or 10, 50, or 100 μM 4-F-GlcNAc were immunoprecipitated with anti-PSGL-1 mAb KPL-1 or isotype IgG control. Immunoprecipitates were resolved on reducing 4–20% SDS-PAGE gradient gels and immunoblotted with mAb HECA-452, E-selectin-Ig, mAb CHO-131, or mAb KPL-1. In (**b**), densitometric analysis of HECA-452, E-selectin-Ig, CHO-131, and KPL-1 immunostained protein from 250 to 100 kDa was performed using NIH ImageJ software. Mean gray scale units were normalized to background staining intensities and averaged from a minimum of three experiments. (*$P<0.01$; statistically significant difference compared with PBS control, Student's paired *t*-test).

owing to the direct incorporation of 4-F-GlcNAc into PSGL-1 *O*-glycans (Dimitroff *et al.*, 2003a). As CLA expression is associated with the progression of cutaneous lymphomas, we hypothesized that CLA modulation could be an attractive approach for altering the pathogenesis of cutaneous lymphomas by preventing the dermal tropism of malignant T cells.

Assaying E-selectin-binding determinants on T cell PSGL-1 with mAb HECA-452 has previously been employed for the analysis of 4-F-GlcNAc efficacy. As sialyl Lewis X on PSGL-1 *O*-glycans also corresponds to P-selectin ligand activity, we have speculated that reductions in HECA-452 antigen expression also reasoned for P-selectin ligand downregulation caused by 4-F-GlcNAc (Dimitroff *et al.*, 2003a). However, a recent description of mAb CHO-131, which specifically binds a core 2 *O*-glycan with a single *N*-acetyllactosamine unit terminated by sialyl Lewis X, suggests that CHO-131 antigen directly correlates with P-selectin ligand expression and is found on only a subset of HECA-452$^+$ T cells (Walcheck *et al.*, 2002). This evidence, therefore, suggests that CHO-131 antigen may be a more suitable marker for analysis of 4-F-GlcNAc efficacy of P-selectin ligand activity. In this report, we analyzed the efficacy and selectivity of 4-F-GlcNAc treatment on E- and P-selectin ligand activities expressed by malignant CLA$^+$ T cells. We also examined the expression of HECA-452 and CHO-131 antigen on PSGL-1 to determine whether E- and P-selectin-binding determinants were differentially inhibited by 4-F-GlcNAc treatment. Effects on sialylated *O*-glycosylations expressed by CD43 were also assayed to elucidate the selectively of 4-F-GlcNAc on other heavily *O*-glycosylated glycoproteins.

Using malignant human CD4 and CLA$^+$ Hut 78 T cells as a model system and non-growth inhibitory concentrations of 4-F-GlcNAc and other control glycosylation treatments, we found that 4-F-GlcNAc exhibited disparate inhibitory potencies on E- and P-selectin ligand activities. E-selectin-binding determinants on Hut 78 cells displayed by the major glycoprotein carrier PSGL-1 were notably downregulated with 50 μM 4-F-GlcNAc, whereas expression of HECA-452 antigen and E-selectin-binding determinants on glycolipid were unaffected. As a result, Hut 78 cellular E-selectin ligand activity was inhibited maximally by 50% of untreated control

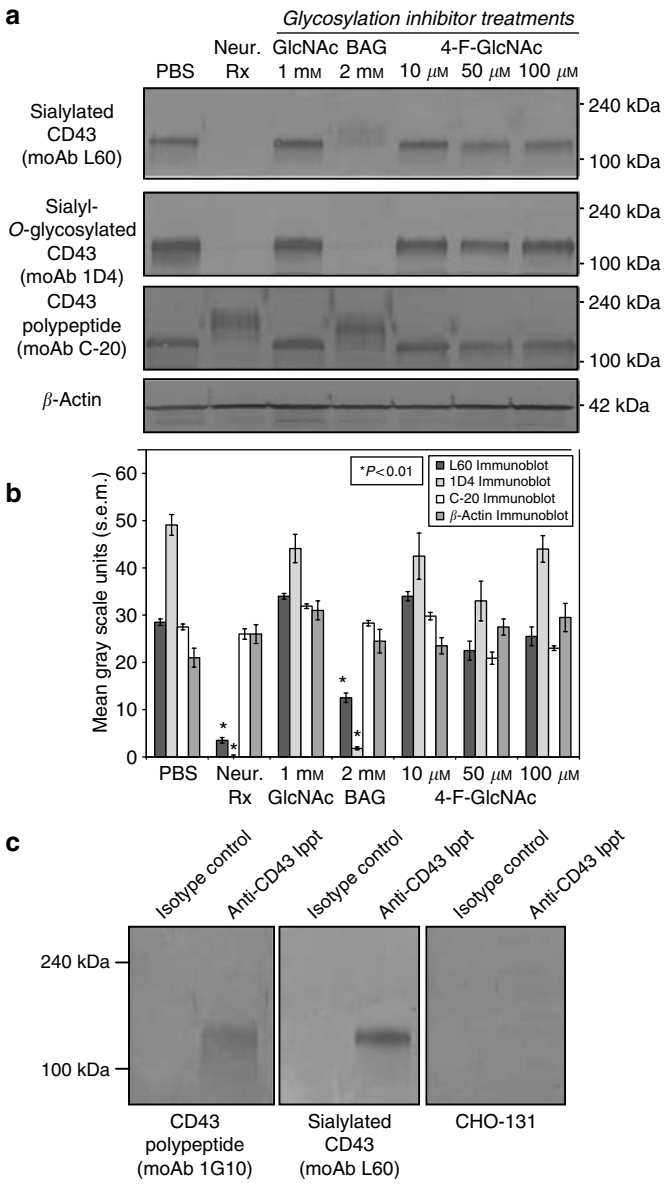

Figure 5. Western blot analysis of sialylated *O*-glycans on CD43 expressed by malignant T cells treated with 4-F-GlcNAc. In (**a**), lysates (5 μg/lane) from Hut 78 cells treated with diluent control, neuraminidase, 1 mM GlcNAc, 2 mM BAG, or 10, 50, or 100 μM 4-F-GlcNAc were resolved on reducing 4–20% SDS-PAGE gradient gels and immunoblotted with anti-CD43 mAb L60. In (**b**), densitometric analysis of L60 immunostained protein from 200 to 100 kDa was performed using NIH ImageJ software. Mean gray scale units (SEM) were normalized to background staining intensities and averaged from a minimum of three experiments. (*$P<0.01$; statistically significant difference compared with PBS control, Student's paired *t*-test) In (**c**), Hut 78 cell lysates were immunoprecipitated with mAb 1G10 and blotted with either mAbs 1G10, L60, or CHO-131. Mouse IgG isotype control immunoprecipitations were performed in parallel and did not show any anti-CD43 mAb reactivity. Experiments were repeated a minimum of three times.

following 4-F-GlcNAc treatment. This moderate reduction in cellular E-selectin ligand activity corroborates with prior data on the resistance of E-selectin glycolipid ligand on normal CLA^+ T cells to 4-F-GlcNAc treatment (Dimitroff *et al.*, 2003a). Interestingly, a recent report suggests that a large percentage CLA^+ T cells also express sialyl 6-sulfo Lewis X, which may be correlative with T cell resistance to 4-F-GlcNAc inhibitory on HECA-452 antigen and E-selectin ligand expression (Ohmori *et al.*, 2006). On the other hand, 4-F-GlcNAc efficacy on CHO-131 antigen and P-selectin ligand expression was more effective, causing complete removal of antigen/activity expression at 50 μM 4-F-GlcNAc. Even at a 5-fold lower concentration of 10 μM, 4-F-GlcNAc significantly blunted CHO-131 antigen on PSGL-1 and P-selectin ligand activity.

As P-selectin carbohydrate recognition determinants were more sensitive than E-selectin carbohydrate recognition determinants to metabolic inhibition by 4-F-GlcNAc, we speculate that CHO-131 antigen on PSGL-1 is a more sensitive carbohydrate moiety than HECA-452 antigen. Whereas HECA-452 antigen may potentially be displayed on multiple sialyl Lewis X-bearing *O*-glycans on PSGL-1 (Wilkins *et al.*, 1996), CHO-131 antigen appears to be restricted to Thr-57 of the N-terminus and P-selectin-binding region of PSGL-1 and shown to contain sialyl Lewis X on a single *N*-acetyllactosamine and not on a poly-*N*-acetyllactosamine (Liu *et al.*, 1998; Leppanen *et al.*, 2002; Walcheck *et al.*, 2002). As sialyl Lewis X moieties have been estimated to be found largely on poly-*N*-acetyllactosamines on PSGL-1 (Wilkins *et al.*, 1996), we have previously argued that 4-F-GlcNAc inhibitory efficacy on HECA-452 antigen displayed by PSGL-1 was owing to the termination of poly-*N*-acetyllactosamines on *O*-glycans (Dimitroff *et al.*, 2003a). However, the data presented here suggest that, in addition to inhibition of poly-*N*-acetyllactosamines bearing HECA-452 antigen, 4-F-GlcNAc could also, and perhaps even preferentially, modify CHO-131 antigen, which contains a single GlcNAc sugar for substitution by 4-F-GlcNAc. Furthermore, in prior studies investigating *in vivo* inhibitory efficacy of 4-F-GlcNAc on E-selectin ligand expression and allergic contact dermatitis, we may not have fully appreciated 4-F-GlcNAc effects on P-selectin ligand expression (Dimitroff *et al.*, 2003b). Complementary inhibition of both E- and P-selectin ligand activities may be responsible for the potent anti-inflammatory effects by 4-F-GlcNAc (Dimitroff *et al.*, 2003b). Indeed, deletion of either E-selectin or P-selectin expression is not sufficient to inhibit delayed-type contact hypersensitivity, whereas E-/P-selectin doubly deficient mice exhibit significantly impaired delayed-type contact hypersensitivity responses (Staite *et al.*, 1996). Thus, we speculate that dual inhibitory efficacy on malignant T cell E- and P-selectin ligand activity would need to be attained to effectively blunt dermal tropism.

To address the selectivity of 4-F-GlcNAc inhibition on selectin ligand biosynthesis among other *O*-glycosylated membrane proteins, we analyzed the expression of sialylated O-glycans on CD43 from 4-F-GlcNAc-treated mice. At concentrations that downregulated CHO-131 and HECA-452 expression on PSGL-1, 4-F-GlcNAc did not appreciably inhibit sialylated *O*-glycan formation on CD43, as determined by the immunoblotting CD43 with anti-CD43 mAb L60. Furthermore, we did not detect CHO-131 antigen on CD43, which corroborates prior evidence showing that CD43

does not bind P-selectin (Moore *et al.*, 1994; Fuhlbrigge *et al.*, 2006) and that CD43 *O*-glycans are mainly non-fucosylated (Maemura and Fukuda, 1992). The resistance of CD43 sialylated *O*-glycans to 4-F-GlcNAc modulation is most likely owing to the lower content of GlcNAc compared with that found in PSGL-1 *O*-glycans. Relative to GalNAc, GlcNAc levels in PSGL-1 *O*-glycans are 2-fold greater than those found on CD43 *O*-glycans (Wilkins *et al.*, 1996). Although CD43 and PSGL-1 share similar levels of core 2 *O*-glycans, generation of sialyl Lewis X conferring selectin-binding determinants is restricted to certain membrane scaffolds that seem to be particularly sensitive to 4-F-GlcNAc modulation.

Collectively, our results reveal that P-selectin ligand modification on malignant T cells is sensitive to 4-F-GlcNAc treatment. Whereas 50 μM 4-F-GlcNAc causes a 50% decrement in P-selectin ligand activity on normal CLA^+ T cells (Dimitroff *et al.*, 2003a), we found that a 5-fold lower concentration of 4-F-GlcNAc inhibits P-selectin ligand activity by 50% on malignant T cells. Whether a similar level of 4-F-GlcNAc inhibitory efficacy can be observed on malignant T cells in patients is still uncertain. The relative level of E- and P- selectin ligand expression and sensitivity of ligand biosynthesis to 4-F-GlcNAc by malignant T cells, *in vivo*, has not yet been ascertained. Despite the lack of *in vivo* data, we speculate that interference of CLA and CHO-131 antigen expression will modify the skin-homing capacity of CTCL cells and suppress the progression of CTCL. In prospective combined treatment strategies, inhibiting the migration of malignant T cells to skin with 4-F-GlcNAc could help retain malignant cells in circulation, whereby other systemic therapies, such as extracorporeal photochemotherapy, could more effectively target circulating malignant cells.

MATERIALS AND METHODS

Cells, enzymes, and metabolic inhibitors

Human Hut 78 T cells, a malignant T cell clone with skin-homing receptor expression from a patient with CTCL, was purchased from ATCC® (Manassas, VA) and maintained in Iscove's modified Dulbecco's medium (ATCC) supplemented with 20% fetal bovine serum/1% penicillin-streptomycin. CLA expression on these cells was determined by flow cytometry prior to metabolic inhibitor studies, and cultures were maintained for $\leqslant$50 passages. *Vibrio cholerae* neuraminidase was purchased from Roche Diagnostics, Inc. (Indianapolis, IN). Protease bromelain, GlcNAc, and BAG were purchased from Sigma Chemical Inc. (St Louis, MO). 4-F-GlcNAc was synthesized and provided by the Chemical Resource Laboratory at Roswell Park Cancer Institute (Buffalo, NY).

Cell treatments

For metabolic inhibitor treatments, exponentially growing Hut 78 cultures were incubated with non-growth inhibitory concentrations of 4-F-GlcNAc (10-100 μM), a putative inhibitor of *N*-acetyllactosamine synthesis), BAG (2 mM) (a metabolic inhibitor of *O*-glycan biosynthesis), and GlcNAc (a molecular control). Non-toxic and carbohydrate-modifying concentrations of 4-F-GlcNAc were chosen based on prior experimentation with treatments of *ex vivo* human T cells and concentrations of BAG were selected based on maximal *O*-glycan inhibitory effects at non-growth inhibitory concentrations as reported previously (Dimitroff *et al.*, 2003a). Cultures were incubated for >3-cell doublings and then harvested for analyses. Cell viability was confirmed by Trypan blue exclusion.

For de-sialylation of cell surface glycoconjugates and removal of all selectin ligand expression, cells were harvested, washed twice with PBS containing Ca^{2+}/Mg^{2+}, and then incubated with 0.2 U/ml neuraminidase for 1 hour at 37°C. For removal of selectin glycoprotein ligands, T cells were incubated with 0.1% bromelain for 1 hour at 37°C as described previously (Dimitroff *et al.*, 2003a). Bromelain is a protease known to remove membrane proteins, including all E-, P-, and L-selectin glycoprotein ligands expressed on human hematopoietic and carcinomatous cells (Dimitroff *et al.*, 2004). Residual E-selectin ligand activity after bromelain treatment would be indicative of activity contributed by a glycolipid component. To confirm complete digestion with neuraminidase and bromelain treatment, we assayed the cell surface expression of sialyl Lewis X and PSGL-1, respectively, by flow cytometry.

Flow cytometry

Cells following inhibitor/protease treatments were washed twice with cold PBS/2% fetal bovine serum and suspended in PBS/1% fetal bovine serum. Rat IgM anti-sialyl Lewis X mAb HECA-452 (20 μg/ml) (BD Biosciences Inc., San Jose, CA), mouse IgG anti-human PSGL-1 mAb PL-2 (20 μg/ml) (BD Biosciences), or mouse IgM anti-sialyl-fucosylated core 2 *O*-glycan mAb CHO-131 (20 μg/ml) (Walcheck *et al.*, 2002), or appropriate isotype-matched control antibody (2 μg/test) was incubated with cells for 1 hour on ice as described previously (Ferenczi *et al.*, 2002; Dimitroff *et al.*, 2003a). Following two washes, cells were incubated with fluorochrome-conjugated secondary antibody for 30 minutes on ice. After washing twice, flow cytometry was performed using a FACScan apparatus equipped with an argon laser tuned at 488 nm (Becton Dickinson). Cells stained with relevant isotype control antibody were subtracted from cells stained with test antibody to control for nonspecific binding.

Parallel-plate flow analysis

Tethering and rolling of T cells on recombinant human E- and P-selectin-Ig chimera (R & D Systems Inc., Minneapolis, MN) were analyzed using in the parallel-plate flow chamber under physiologic shear stress conditions (Dimitroff *et al.*, 2003a). For preparation of immobilized chimera spots, E-selectin-Ig (50 ng/50 μl 0.1 M $NaHCO_3$, pH 9.6) or P-selectin-Ig (50 ng/50 μl 0.1 M $NaHCO_3$, pH 9.6) was pipetted onto Ten-twenty-nine™ Petri dishes and allowed to adsorb for 24 hours at 4°C. Human IgG (500 μg/50 μl spots) were also prepared to control for the contribution of human IgG in chimera. After removing chimeric solution, fetal bovine serum (100%) was then pipetted over the chimera spots and incubated for 2 hours at 37°C to block nonspecific binding sites. Untreated or treated T cell cultures as described above were washed twice in Hank's balanced salt solution, suspended at 2×10^6/ml in Hank's balanced salt solution /10 mM HEPES/2 mM $CaCl_2$ (H/H/Ca^{2+}). and infused into the chamber over chimera or human IgG spots. Cell tethering was permitted at 0.6 Dyn/cm^2 for 1 minute, then stepwise increments in shear stress every 15 seconds were employed to a final shear stress level of 60 Dyn/cm^2. Cell rolling assessments were made at 1.5 Dyn/cm^2 from the mid-point of the chamber viewing field (4 fields/selectin spot, three different experiments) at $\times$200 magnification. All experiments were observed in real time and videotaped for

offline analysis. Negative control experiments were performed in parallel, wherein cell binding was examined in H/H adhesion assay medium containing 5 mM EDTA to chelate Ca^{2+} required for selectin binding as well as assaying cell binding to human IgG control spots.

Cell lysate preparation, immunoprecipitation, SDS-PAGE, and Western blotting

Cell lysates were prepared and analyzed as follows. Cells from metabolic inhibitor/enzymatic treatments were washed 3 × in ice-cold PBS and lysed in buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.02% NaAzide, 20 μg/ml phenylmethyl-sulfonyl fluoride, Complete™ protease inhibitor tablets (Roche Diagnostics, Inc.) and 2% NP-40 at a ratio of 250 μl lysis buffer to 10^8 cells. Following 2 hours incubation on ice, insoluble cellular debris was pelleted by centrifugation for 30 minutes at 10,000 r.p.m. Solubilized protein was harvested and quantified by Bradford protein assay (Sigma Chemical).

For immunoprecipitation of PSGL-1 or CD43, anti-human PSGL-1 mAb KPL-1 or anti-human CD43 mAb 1G10 (BD Biosciences) was added to cell lysates pre-cleared in recombinant protein G-agarose (Invitrogen, Inc., Carlsbad, CA) at a ratio of 2 μg Ab to 100 μg lysate for 18 hours at 4°C on a rotator. Immunoprecipitations with mouse IgG isotype control at a similar Ab:lysate ratio were also performed to serve as negative controls. The antibody–lysate mixture was then added to protein G-agarose, which was pre-washed with lysis buffer/2% NP-40/1% SDS/1% BSA, and incubated for >2 hours at 4°C on a rotator. The antibody–lysate–protein G-agarose mixtures were spun down, supernatants were removed, and immunoprecipitates were washed 3 × with lysis buffer/2% NP-40/1% SDS/1% BSA and then twice with lysis buffer/2% NP-40.

For SDS-PAGE and Western blotting, total cell lysates or immunoprecipitates were diluted and boiled in reducing sample buffer, and separated on 4–20% gradient SDS-PAGE gels. Resolved protein was transferred to Immunoblot™ PVDF membrane (Bio-Rad Inc., Hercules, CA) and blocked with fetal bovine serum for 1 hour at room temperature. Blots were incubated with anti-human CLA mAb HECA-452 (1 μg/ml), anti-human PSGL-1 mAb KPL-1 (1 μg/ml), mouse E-selectin-human Ig chimera, anti-sialyl-fucosylated core 2 *O*-glycan mAb CHO-131 (1 μg/ml), anti-human CD43 mAb 1G10, or mouse IgG anti-human CD43 mAb L60 (1 μg/ml) for 1 hour at room temperature. Isotype control immunoblots using either rat IgM, human IgG, mouse IgM, or mouse IgG were performed in parallel to control for primary antibody specificity. After three washes with Tris-buffered saline/0.1%Tween-20 (10 minutes/wash), blots were incubated with alkaline phosphate-conjugated goat anti-rat IgM (1:400), rat anti-mouse IgM (1:1000), goat anti-human IgG (1:1000), or goat anti-mouse IgG (1:5000). Alkaline phosphate substrate, Western Blue® (Promega, Madison, WI) was then added to develop blots.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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