Sialyltransferase inhibition leads to inhibition of tumor cell interactions with E-selectin, VCAM1, and MADCAM1, and improves survival in a human multiple myeloma mouse model

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ABSTRACT

Aberrant glycosylation resulting from altered expression of sialyltransferases, such as ST3 β-galactoside α2-3-sialyltransferase 6, plays an important role in disease progression in multiple myeloma (MM). Hypersialylation can lead to increased immune evasion, drug resistance, tumor invasiveness, and disseminated disease. In this study, we explore the *in vitro* and *in vivo* effects of global sialyltransferase inhibition on myeloma cells using the pan-sialyltransferase inhibitor $3F_{ax}$-Neu5Ac delivered as a per-acetylated methyl ester pro-drug. Specifically, we show *in vivo* that $3F_{ax}$-Neu5Ac improves survival by enhancing bortezomib sensitivity in an aggressive mouse model of MM. However, $3F_{ax}$-Neu5Ac treatment of MM cells *in vitro* did not reverse bortezomib resistance conferred by bone marrow (BM) stromal cells. Instead, 3Fax-Neu5Ac significantly reduced interactions of myeloma cells with E-selectin, MADCAM1 and VCAM1, suggesting that reduced sialylation impairs extravasation and retention of myeloma cells in the BM. Finally, we showed that $3F_{ax}$-Neu5Ac alters the post-translational modification of the α4 integrin, which may explain the reduced affinity of α4β1/α4β7 integrins for their counter-receptors. We propose that inhibiting sialylation may represent a valuable strategy to restrict myeloma cells from entering the protective BM microenvironment, a niche in which they are normally protected from chemotherapeutic agents such as bortezomib. Thus, our work demonstrates that targeting sialylation to increase the ratio of circulating to BM-resident MM cells represents a new avenue that could increase the efficacy of other anti-myeloma therapies and holds great promise for future clinical applications.

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Introduction

Multiple myeloma (MM) is characterized by clonal expansion of malignant plasma cells in the bone marrow (BM). Despite significant advances in treatment, MM remains incurable, with drug resistance mediated by the BM microenvironment being an important contributory factor.[1,2] A related remarkable feature of MM is the ability for MM cells to spread from one BM site to another, which implies a persistent trafficking of circulating MM cells into and out of the BM microenvironment.[3,4]

Homing into the BM is physiologically governed by a diverse array of molecules such as Stromal cell-derived factor 1α (SDF1α), E-selectin, and various integrin co-receptors including Mucosal vascular addressin cell adhesion molecule 1 (MADCAM1).[5] In the context of MM, SDF1α plays a major role in migration, adhesion, homing, and possibly retention of MM cells in the BM.[6-9] Mediators of SDF1α activ-

ity in MM include matrix metalloproteinase and integrin $\alpha4\beta1$-dependent adhesion on fibronectin and Vascular cell adhesion molecule 1 (VCAM1).[10-12] Recently, E-selectin has also been shown to play a role in homing and retention of MM cells in the BM.[13,14] In particular, we have shown that sialofucosylated structures recognized by E-selectin, such as Sialyl Lewis[a/x] (SLe[a/x]), enable MM cells to escape the cytotoxic effects of bortezomib *in vivo* most likely by hiding in the BM.[14] Indeed, MM cells enriched for E-selectin ligands recognized by the monoclonal antibody Heca452, were resistant to bortezomib treatment *in vivo* and this resistance was reversed by a small glycomimetic molecule GMI-1271, which inhibits the interaction between E-selectin and E-selectin ligands.[14] Thus, SDF1α and E-selectin may act co-operatively to allow extravasation of MM cells into the BM niche where they can proliferate and evade drug treatments.

Post-translational glycosylation of proteins and lipids plays many physiological and pathophysiological roles. There is a growing appreciation that aberrant glycosylation is considered a hallmark of cancer,[15,16] with one of the most prominent changes being a role for hypersialylation as a driver of tumor progression, metastasis and invasion.[17,18] Hypersialylation is largely the result of overexpression of sialyltransferases (STs), which catalyze the attachment of sialic acids *via* different glycosidic linkages ($\alpha2$-3, $\alpha2$-6, or $\alpha2$-8) to the underlying glycan chain.[17,19] We have previously established an important role for aberrant sialylation in homing and survival in MM.[20] Specifically, high expression of the ST3 β-galactoside $\alpha2$-3-sialyltransferase 6 (ST3GAL6) in MM cell lines and patient samples is associated with inferior outcomes. Knocking down ST3GAL6 reduces sialic acid expression on MM cells, decreasing their ability to home to the BM. Since ST3GAL6 participates in the generation of SLe[a/x] structures, which forms the minimal E-selectin ligand, and may also be involved in sialylation of other structures important in MM homing and adhesion,[21-23] we sought to investigate if we could therapeutically target sialylation on MM cells, and whether this would affect BM homing and survival in mice.

Here we show that pre-treatment of MM cells enriched for E-selectin ligands with $3F_{ax}$-Neu5Ac, a global inhibitor of the ST family,[24] significantly reduces cell surface sialylation of these cells, prolongs survival in xenograft mice and enhances their *in vivo* sensitivity to bortezomib. *In vitro*, $3F_{ax}$-Neu5Ac impairs the interaction between MM cells and E-selectin under shear stress and, surprisingly, also greatly reduces their interaction with VCAM1 and MADCAM1 under similar conditions. In this respect, we show that 3Fax-Neu5Ac alters the post-translational modification of integrin $\alpha4$ on MM cells. This implies a dual effect on homing, whereby blockade of selectin ligands and integrin-mediated interactions with BM endothelial cells prevents extravasation of MM cells in the BM. Our results suggest great potential for improved patient outcomes by targeting sialylation on MM cells, especially when used in combination with other active MM agents.

Methods

Selection of E-selectin ligand-enriched cells

The E-selectin ligand-enriched MM1S cell line (MM1S[Heca452]) was generated from GFP[+]/Luc[+] MM1S and parental MM1S cell lines by two rounds of fluorescently-activated cell sorting (FACS) using the fluorescent Heca452 antibody (Biolegend; San Diego, CA, USA). Cells were maintained in RPMI-1640 (VWR; Radnor, PA, USA) containing L-glutamine, 10% heat inactivated fetal bovine serum (HI-FBS, VWR), and 1X antibiotic-antimycotic (Corning; Kennebunk, ME, USA).

Animal experiments

All experimental studies and procedures involving mice were performed in accordance with protocols approved by the governing Institutional Animal Care and Use Committee (IACUC) and all state and federal laws. In the toxicity study, 8-week old male and female C57BL/6J mice (n=8) received 0, 6.25, 12.5 or 25 mg/kg body weight doses of $3F_{ax}$-Neu5Ac (EMD Millipore; Burlington, USA) delivered intraperitoneally (i.p.) once daily for seven days. The drug was dissolved in dimethyl sulfoxide (DMSO) (VWR) and subsequently diluted 2-fold in PEG-300 (Sigma Aldrich; St. Louis, MO, USA). Mice were monitored daily for signs of discomfort, especially at the site of injection. In the homing study, 6-week old female Fox Chase SCID-Beige mice (Charles River Laboratory; Wilmington, MA, USA) (n=9 or 10) were inoculated *via* tail vein injections with $5x10^6$ Heca452-enriched GFP[+]/Luc^{+} MM1S cells, which had been pre-treated with either vehicle or 300 μM $3F_{ax}$-Neu5Ac for seven days in culture before inoculation. Starting one day post inoculation, mice received either vehicle (PBS) or bortezomib (Selleck; Houston, TX, USA) injections intraperitoneally twice weekly. To monitor toxicity, mice were weighed twice weekly. Mice were frequently monitored for clinical signs of treatment-related side effects. Survival end points were mouse death or euthanasia as required by the IACUC (a single observation of >30% body weight loss, 3 consecutive measurements of >25% body weight loss, or impaired hind limb use). Survival differences were analyzed by the Kaplan-Meier method.

Bioluminescent imaging

Starting on day 7, and biweekly until day 30, tumor burden was assessed with bioluminescence imaging (BLI) in an IVIS® Lumina LT (Perkin Elmer Inc.; Waltham, MA, USA) equipped with a CCD camera (cooled at -90°C), mounted on a light-tight specimen chamber. Mice were injected with 150 mg/kg i.p. filter-sterilized D-luciferin substrate (VivoGlo, Promega; Madison, WI, USA) and imaged after 10 minutes. Data were acquired and analyzed using LivingImage software 4.5.1. (PerkinElmer). BLI flux equaling the radiance (photons/s) in each pixel integrated over the region of interest (ROI) area (cm^2), where the ROI was the whole mouse, was used to quantify tumor burden. BLI and mouse weight data were graphed and analyzed only for days in which all mice remained in the study to avoid artifacts due to mouse death.

Statistical analysis

All data are expressed as mean±standard error of the mean (SEM), unless otherwise noted. Student's *t*-test, ordinary one-way or two-way ANOVA tests were used to determine significance, using $P<0.05$ as the cut-off, with Tukey's multiple comparison *post-hoc* testing unless otherwise noted. **** $P<0.0001$; *** $P<0.001$; ** $P<0.01$; * $P<0.05$. GraphPad Prism 6.02 software (La Jolla, CA, USA) was used to compute all statistical calculations unless otherwise noted.

Additional information concerning materials and methods can be found in the *Online Supplementary Appendix*.

Results

Treatment of mice with 3F$_{ax}$-Neu5Ac causes a dose dependent decrease in sialoside expression on multiple organs systemically

Building upon the previous 3F$_{ax}$-Neu5Ac *in vivo* experience,[25,26] we first studied the effects of global desialylation *in vivo* after systemic administration of 3F$_{ax}$-Neu5Ac. Mice were treated with 6.25, 12.5, and 25 mg/kg of 3F$_{ax}$-Neu5Ac daily for seven consecutive days. Sialylation was monitored using two different lectins after seven days of treatment: the *Sambucus nigra* lectin (SNA), which binds α2-6 linked sialic acids, and the *Peanut agglutinin* lectin (PNA), which binds to desialylated T antigen. In mice treated with 25 mg/kg 3F$_{ax}$-Neu5Ac, there was a clear decrease in SNA staining in the kidney, spleen and liver (*Online Supplementary Figure S1A-C*), consistent with a reduction in α2-6 linked sialic acid expression. Moreover, at the same dose, there was a contemporary increase in PNA staining (*Online Supplementary Figure S2A-C*) consistent with decreased sialic acid expression leading to exposure of terminal galactose residues, such as the T antigen (Galβ1-3GalNAcαSer/Thr). To determine the effects of 3F$_{ax}$-Neu5Ac treatment on sialylation of cells of the immune system, peripheral blood B cells were used as representative immune cells. The median fluorescent intensity (MFI) values for SNA and PNA positive staining were determined on the seventh day of treatment and the seventh day after the final treatment. Similar to what was observed in histology sections, 3F$_{ax}$-Neu5Ac treatment induced a decrease in the SNA MFI with the highest dose (25 mg/kg) having a significant fold change compared to the control at the seventh day of dosing (Figure 1A). As expected, the PNA lectin MFI significantly increased with 3F$_{ax}$-Neu5Ac treatment by the final day of treatment as well (Figure 1C). Sialic acid expression remained low after seven days of recovery after the 25 mg/kg dose, as particularly evident in cells stained with PNA, although a trend was also observed in the SNA-stained cells (Figure 1B and D), suggesting that recovery takes longer than seven days. Because sialylation is crucial to kidney filtration, we also determined the effects of the dose regimen in the kidneys. H&E staining showed no obvious histological changes in the kidney after seven days of recovery (Figure 1E). However, mice that received the highest dose, 25 mg/kg, did experience edema in the peritoneal cavity, as previously reported.[27] These data demonstrate that 3F$_{ax}$-Neu5Ac can successfully inhibit the expression of sialic acid systemically, but that local BM- or myeloma-specific delivery may be necessary to overcome effects on other organs in future studies.

Treatment of MM1S^{Heca452} with 3F$_{ax}$-Neu5Ac decreases sialylation *in vitro*

We next examined whether 3F$_{ax}$-Neu5Ac could significantly reduce the expression of sialic acid on MM1S^{Heca452} cells. These cells are enriched for E-selectin ligand expression compared to the MM1S parental line and *in vivo* generate a very aggressive disease which displays resistance to bortezomib.[14] The MM1S^{Heca452} cell line has been extensively characterized; their sensitivity to bortezomib,

clonogenic potential, and proliferation *in vitro* are identical to the parental line and their aggressive phenotype becomes evident only *in vivo*.[14] Sialylation was monitored using the Heca452 and CD15s antibodies, which recognize the sialofucosylated structure SLe$^{a/x}$, the Maackia Amurensis Lectin II (MAL II), which preferentially binds to α2-3 linked sialic acid, and SNA. Over a seven day span in culture, 300 μM of 3F$_{ax}$-Neu5Ac significantly decreased the Heca452 staining in a time-dependent manner (*Online Supplementary Figure S3*). After seven days of treatment, 3F$_{ax}$-Neu5Ac decreased the MFI and the total number of the Heca452, CD15s, MALII and SNA positive cells (Figure 2). Importantly, 3F$_{ax}$-Neu5Ac treatment did not induce a significant change in the sensitivity to bortezomib *in vitro* (Figure 4). Based on these data, we chose to pre-treat the MM1S^{Heca452} cells with 300 μM of 3F$_{ax}$-Neu5Ac for seven days to significantly reduce sialylation on the cell surface.

In vivo, pre-treatment of MM1S^{Heca452} cells with 3F$_{ax}$-Neu5Ac reduces tumor burden and increases survival, and co-treatment with bortezomib further enhances these outcomes

To study the impact of global sialylation inhibition specifically on MM cells and to avoid kidney toxicity related to 3F$_{ax}$-Neu5Ac treatment, we pre-treated the MM1S^{Heca452} cells with 300 μM of 3F$_{ax}$-Neu5Ac or vehicle for seven days, inoculated these cells into immunocompromised mice, and followed tumor burden using bioluminescence imaging. Mice inoculated with 3F$_{ax}$-Neu5Ac-pre-treated MM1S^{Heca452} (3F$_{ax}$-Neu5Ac MM1S^{Heca452} mice) showed reduced tumor burden compared to mice inoculated with vehicle-pre-treated MM1S^{Heca452} (vehicle MM1S^{Heca452} mice) (Figure 3A and B). Two cohorts in this study in addition received bortezomib treatment, which decreased the tumor burden in the vehicle and 3F$_{ax}$-Neu5Ac MM1S^{Heca452} mice (Figure 3A and B). The 3F$_{ax}$-Neu5Ac MM1S^{Heca452} mice that received bortezomib treatment had the least tumor burden throughout the study compared to the other groups (Figure 3B). Importantly, even in the absence of bortezomib, pre-treatment of MM1S^{Heca452} cells with 3F$_{ax}$-Neu5Ac reduced tumor burden compared to vehicle MM1S^{Heca452} mice, suggesting that 3F$_{ax}$-Neu5Ac pre-treatment is beneficial even without the addition of chemotherapy. We also observed that the 3F$_{ax}$-Neu5Ac MM1S^{Heca452} mice survived significantly longer than the vehicle MM1S^{Heca452} mice (Figure 3C). Notably, bortezomib treatment did not prolong survival of the vehicle MM1S^{Heca452} mice, confirming our previous observation that in this *in vivo* model, these MM cells are more refractory to bortezomib treatment (Figure 3C).[14] Above all, pre-treatment of MM1S^{Heca452} cells with 3F$_{ax}$-Neu5Ac in combination with bortezomib led to longer survival compared to the other groups, suggesting a synergistic therapeutic effect. No significant difference was observed in change in body weight between the treatment groups (Figure 3D). Overall, we demonstrate that 3F$_{ax}$-Neu5Ac reduces tumor burden and increases survival, and that additional treatment with bortezomib has a synergistic effect with 3F$_{ax}$-Neu5Ac.

3F$_{ax}$-Neu5Ac treatment partially reverts stroma but not endothelial-induced bortezomib resistance *in vitro*

To gain insight into the mechanism(s) of increased bortezomib sensitivity in response to 3F$_{ax}$-Neu5Ac treat-
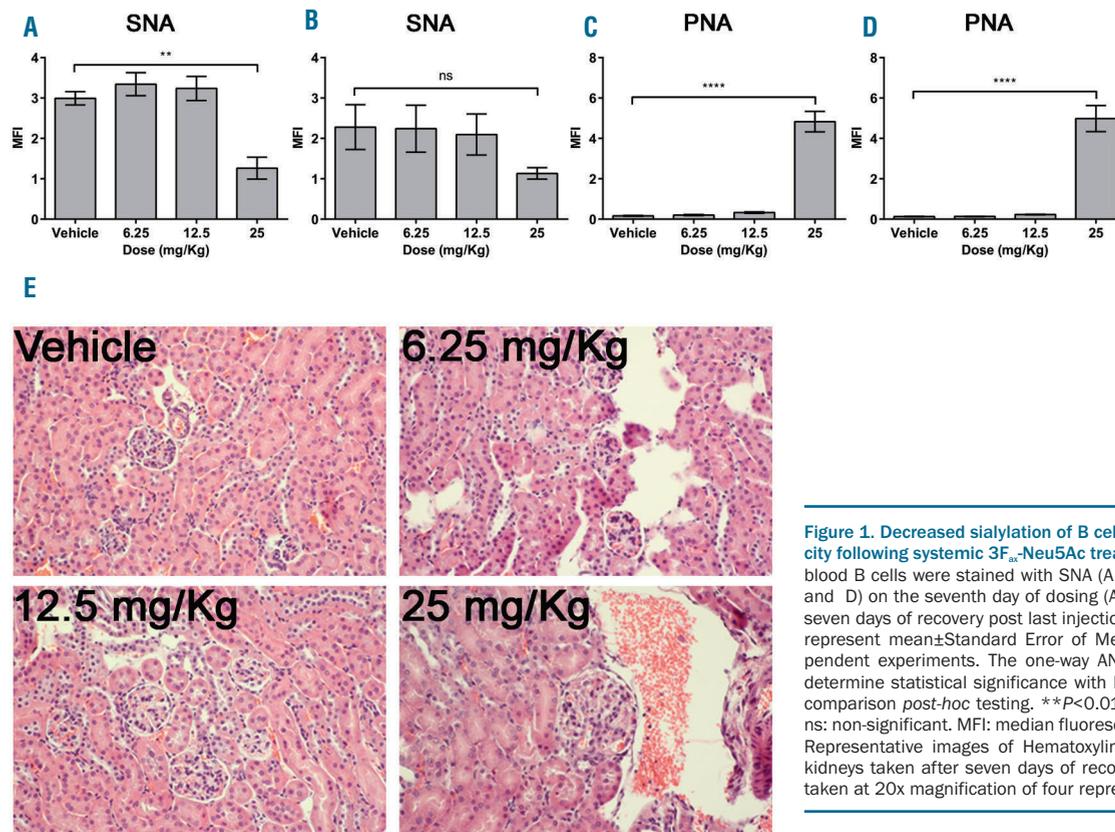
Figure 1. Decreased sialylation of B cells and kidney toxicity following systemic 3F_{ax}-Neu5Ac treatment. Peripheral blood B cells were stained with SNA (A and B) or PNA (C and D) on the seventh day of dosing (A and C) and after seven days of recovery post last injection (B and D). Bars represent mean±Standard Error of Mean of three independent experiments. The one-way ANOVA was used to determine statistical significance with Dunnett's multiple comparison *post-hoc* testing. **$P<0.01$; ****$P<0.0001$; ns: non-significant. MFI: median fluorescence intensity. (E) Representative images of Hematoxylin & Eosin stained kidneys taken after seven days of recovery. Images were taken at 20x magnification of four representative mice.

ment, we sought to investigate *in vitro* the effects of 3F$_{ax}$-Neu5Ac pre-treatment on the sensitivity to bortezomib in MM1S^{Heca452} cells in co-culture conditions that partially recapitulate the BM environment. To this end, we used the well-established HS5 stromal cell line,[28] primary BM stromal cells (BMSC) derived from MM patients, and the BM endothelial cell line BMEC-60. The latter was also treated with 10 ng/mL TNFα for four hours before co-culture to induce activation. BMEC-60, BMSC and, to an even greater extent, HS5 induced resistance to bortezomib (5 nM) in MM1S^{Heca452} cells (Figure 4A-C). 3F$_{ax}$-Neu5Ac pre-treatment caused only minor, although significant re-sensitization to bortezomib in the presence of HS5 and BMSC (Figure 4A and B) and did not reverse BMEC-60-induced bortezomib resistance (Figure 4C). Importantly, in the absence of BM-derived cells, the 3F$_{ax}$-Neu5Ac pre-treatment had only a minor effect on the MM1S^{Heca452} response to bortezomib. Together these data indicate that, in MM cells, bortezomib resistance that is BM stromal cell-driven, although maybe not endothelial cell-driven, can be partially reversed by inhibition of sialylation.

3F$_{ax}$-Neu5Ac treatment does not affect migration in response to SDF1α

Since 3F$_{ax}$-Neu5Ac did not completely reverse bortezomib resistance induced by BM cell lines and patient-derived-BMSC *in vitro*, we reasoned that the mechanism(s) of bortezomib re-sensitization *in vivo* induced by 3F$_{ax}$-Neu5Ac may also involve a defect in the ability of the MM1S^{Heca452} cells to home into the protective BM microenvironment. To explore this possibility, we first examined the effects of 3F$_{ax}$-Neu5Ac pre-treatment on migration in

response to SDF1α in a transwell assay. DMSO and 3F$_{ax}$-Neu5Ac pre-treated MM1S^{Heca452} cells showed enhanced migration in response to SDF1α (Figure 5A). However, spontaneous as well as SDF1α-induced migration were similarly inhibited by 3F$_{ax}$-Neu5Ac pre-treatment (Figure 5A). Indeed, when we specifically examined migration in response to SDF1α by subtracting spontaneous migration (no SDF1α) to SDF1α-containing samples, we observed that 3F$_{ax}$-Neu5Ac pre-treatment did not affect SDF1α-driven migration (Figure 5B). These data suggest that 3F$_{ax}$-Neu5Ac pre-treatment has an impact on the motility of the cells but not specifically on SDF1α-induced migration.

3F$_{ax}$-Neu5Ac treatment impairs adhesion and rolling of MM1S^{Heca452} cells on E-selectin, MADCAM1 and VCAM1

We next examined whether 3F$_{ax}$-Neu5Ac could influence adhesion and rolling on selectins and integrin co-receptors important in BM homing.[3-5] To this end, we performed adhesion and rolling assays under shear stress on E-selectin, MADCAM1 and VCAM1-coated substrates. MM1S^{Heca452} cells showed robust interactions with E-selectin which could be subcategorized into firm adhesion and rolling (Figure 6A-C). 3F$_{ax}$-Neu5Ac pre-treatment dramatically impaired this interaction by decreasing the number of adherent cells (Figure 6A and C). The number of rolling cells was not affected (Figure 6B). However, when we looked at the rolling velocity, we observed an increase in the velocity of the MM1S^{Heca452} cells pre-treated with 3F$_{ax}$-Neu5Ac *versus* DMSO controls, indicating a decrease in the affinity of the E-selectin ligands for E-selectin (*Online Supplementary Figure S4A-C*). These data
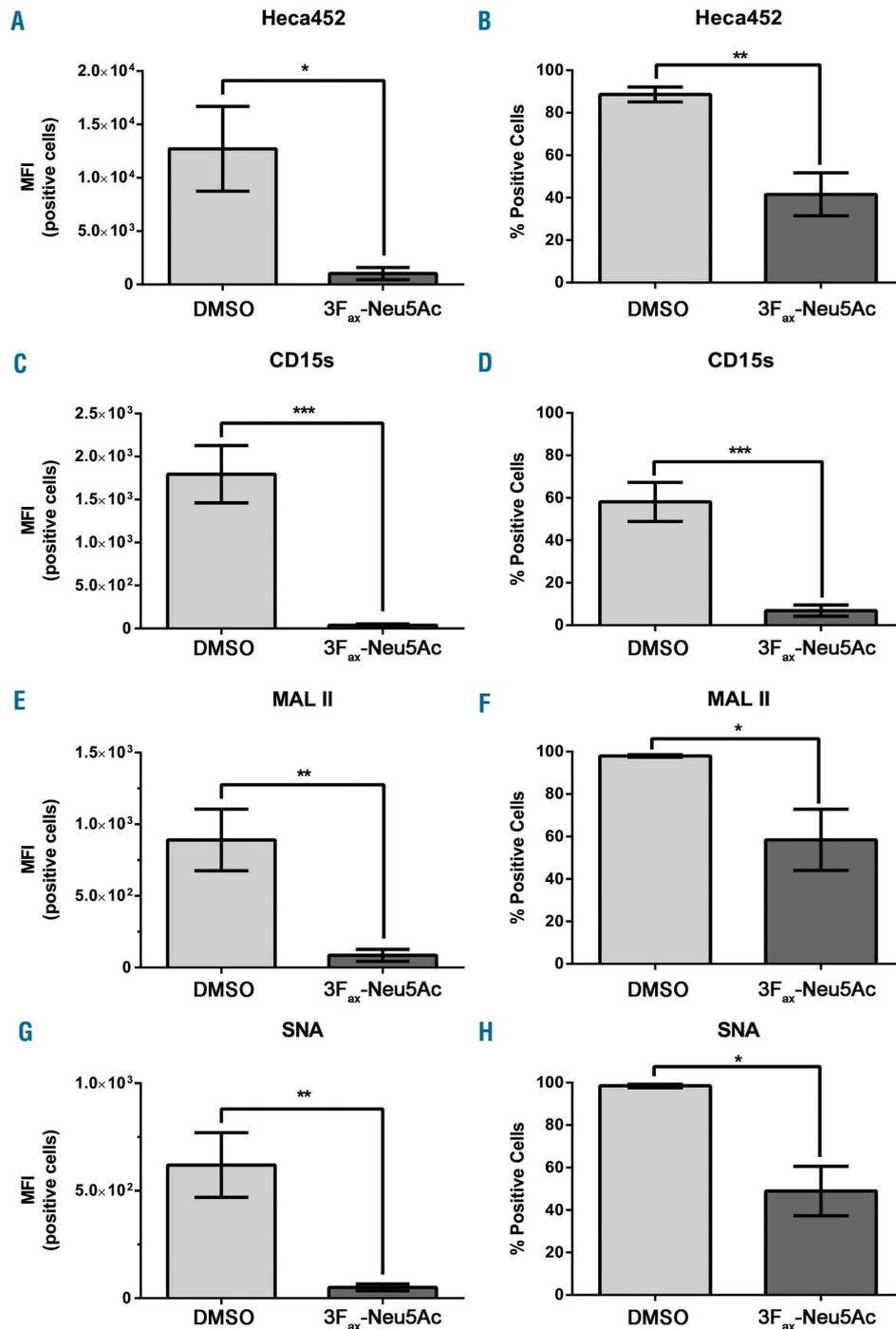
Figure 2. 3F$_{ax}$-Neu5Ac treatment decreases sialylation in the MM1S^{Heca452} cell line. MM1S^{Heca452} cells were treated with 300 μM 3F$_{ax}$-Neu5Ac or dimethyl sulfoxide (DMSO) (vehicle control) for seven days. After treatment, cells were collected and stained with the Heca452 (A and B), CD15s (C and D), MALII (F and G), or SNA (G and H) antibodies or lectins. Bars represent mean±Standard Error of Mean of three independent experiments. Unpaired Student's *t*-test was used to determine statistical significance. *P<0.05; ** P<0.01; ***P<0.001. MFI: median fluorescence intensity.

are consistent with a requirement of sialic acid for E-selectin binding. The MM1S^{Heca452} also showed robust adhesion and rolling on MADCAM1 under shear stress (Figure 6D-F). 3F$_{ax}$-Neu5Ac pre-treatment induced a decrease in the total number of cells interacting with MADCAM1 and, in particular, decreased the number of adherent cells while increasing the number of rolling cells, suggesting a decrease in the affinity of integrin α4β7 for MADCAM1. Again, the velocity of the rolling cells was increased by 3F$_{ax}$-Neu5Ac, indicating a weaker attachment (*Online Supplementary Figure S4D-F*). Finally, the 3F$_{ax}$-Neu5Ac pre-treatment induced a reduction in the number of MM1S^{Heca452} cells interacting with VCAM1,

which was exclusively adhesion (Figure 6G). Indeed, rolling on VCAM1 was not observed indicating strong interactions between integrin α4β1 and VCAM1. Altogether, these data indicate that desialylation of the MM1S^{Heca452} cells impairs interaction (a summation of adhesion and rolling) with E-selectin, MADCAM1 and VCAM1.

### 3F$_{ax}$-Neu5Ac treatment alters the post-translational modifications on integrin α4

Next, we examined whether 3F$_{ax}$-Neu5Ac treatment decreased protein expression of integrins α4β7 or α4β1, which bind VCAM1 and MADCAM1 respectively, on

MM1S[Heca452] cells.[29-31] Flow cytometry analysis revealed that 3F$_{ax}$-Neu5Ac did not decrease α4, β1 or β7 expression (*Online Supplementary Figure S5D-F*). We then investigated whether 3Fax-Neu5Ac affected post-translational modifications on these integrins, which in turn would result in an altered mobility on SDS-PAGE. Indeed, Western blot analysis of integrin α4 revealed a marked shift of the mature as well as the C-terminal cleavage form in response to 3F$_{ax}$-Neu5Ac, suggesting an alteration of α4 post-translational modifications probably due to its desialylation (Figure 7A). To our knowledge, this is the first evidence that integrin α4 is post-translationally sialylated. The integrins β1 and β7 were not heavily affected by

3Fax-Neu5Ac pre-treatment (Figure 7B and C). Similar results were obtained in the parental MM1S cell line (*Online Supplementary Figure S6A-C*). Altogether, these data indicate that 3F$_{ax}$-Neu5Ac primarily alters integrin α4 post-translationally, which most likely results in the observed weaker interaction of the MM cells with MAD-CAM1 and VCAM1.

Discussion

In this study, we examined whether global inhibition of sialylation could increase bortezomib sensitivity in an
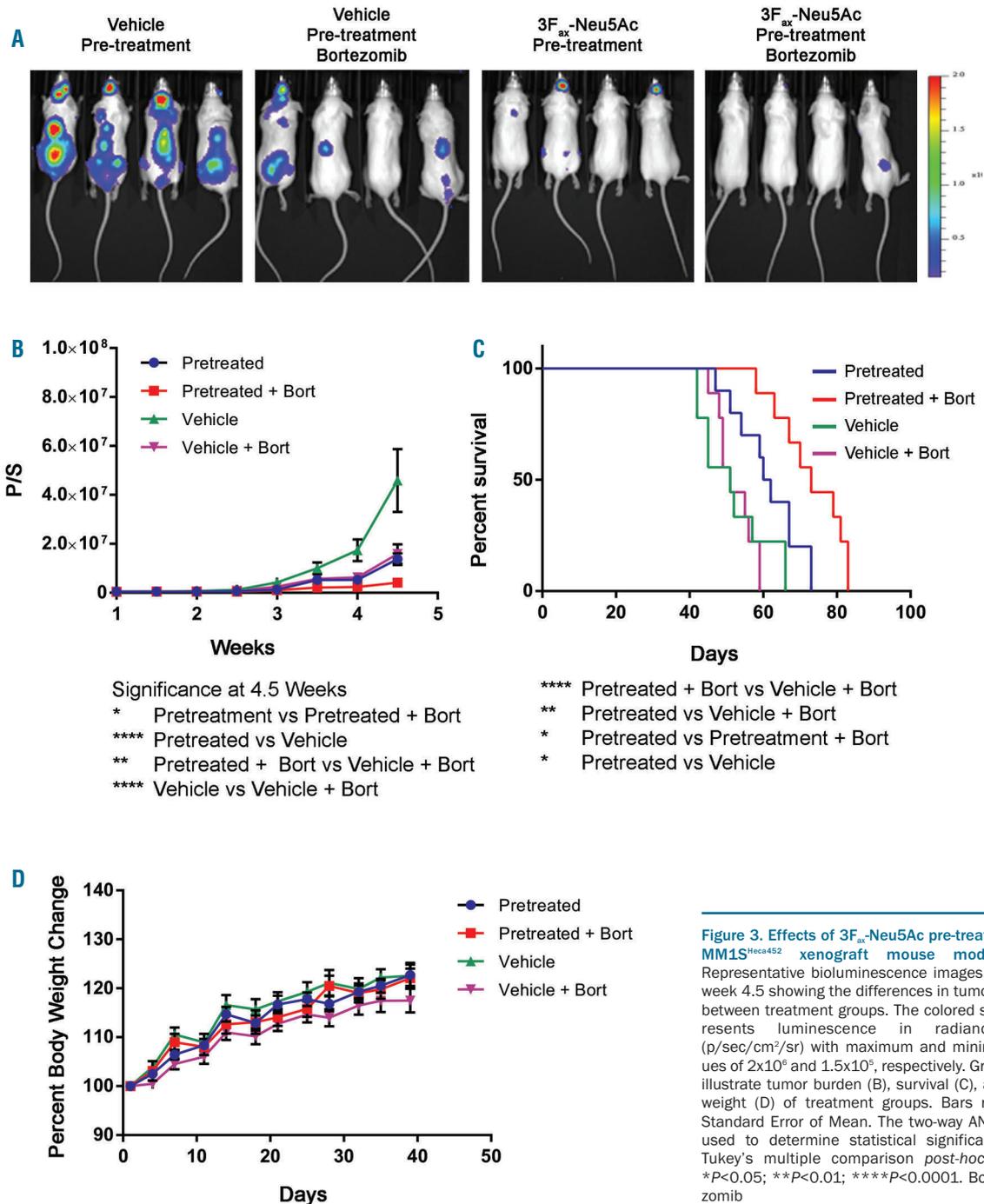
Figure 3. Effects of 3F$_{ax}$-Neu5Ac pre-treatment on MM1S[Heca452] xenograft mouse models. (A) Representative bioluminescence images taken at week 4.5 showing the differences in tumor burden between treatment groups. The colored scale represents luminescence in radiance unit (p/sec/cm^2/sr) with maximum and minimum values of 2x10^6 and 1.5x10^5, respectively. Graph lines illustrate tumor burden (B), survival (C), and body weight (D) of treatment groups. Bars represent Standard Error of Mean. The two-way ANOVA was used to determine statistical significance with Tukey's multiple comparison *post-hoc* testing. *$P<0.05$; **$P<0.01$; ****$P<0.0001$. Bort: bortezomib

aggressive MM mouse model, which employs xenotrans- plantation of MM cells that have been enriched for E- selectin ligands.[14] To this end, we used the pan-sialyltrans- ferase inhibitor $3F_{ax}$-Neu5Ac, which had been previously shown to efficiently block sialylation in leukemic cells.[24]

In a preliminary dose-finding *in vivo* study, we observed that $3F_{ax}$-Neu5Ac decreased sialylation in various tissues, including cells of the immune system. At its effective

dose, 25 mg/kg, $3F_{ax}$-Neu5Ac induced edema in the peri- toneal cavity of mice suggesting that desialylation of the glomerulus could lead to dose-limiting toxicity, as previ- ously reported.[27] To overcome $3F_{ax}$-Neu5Ac-induced kid- ney toxicity and to examine the role of sialylation specifi- cally in MM, we treated the E-selectin enriched MM1S cell line, MM1S[Heca452], with $3F_{ax}$-Neu5Ac before inoculation, an approach that has been successfully used to uncover a crit-

A

B

C
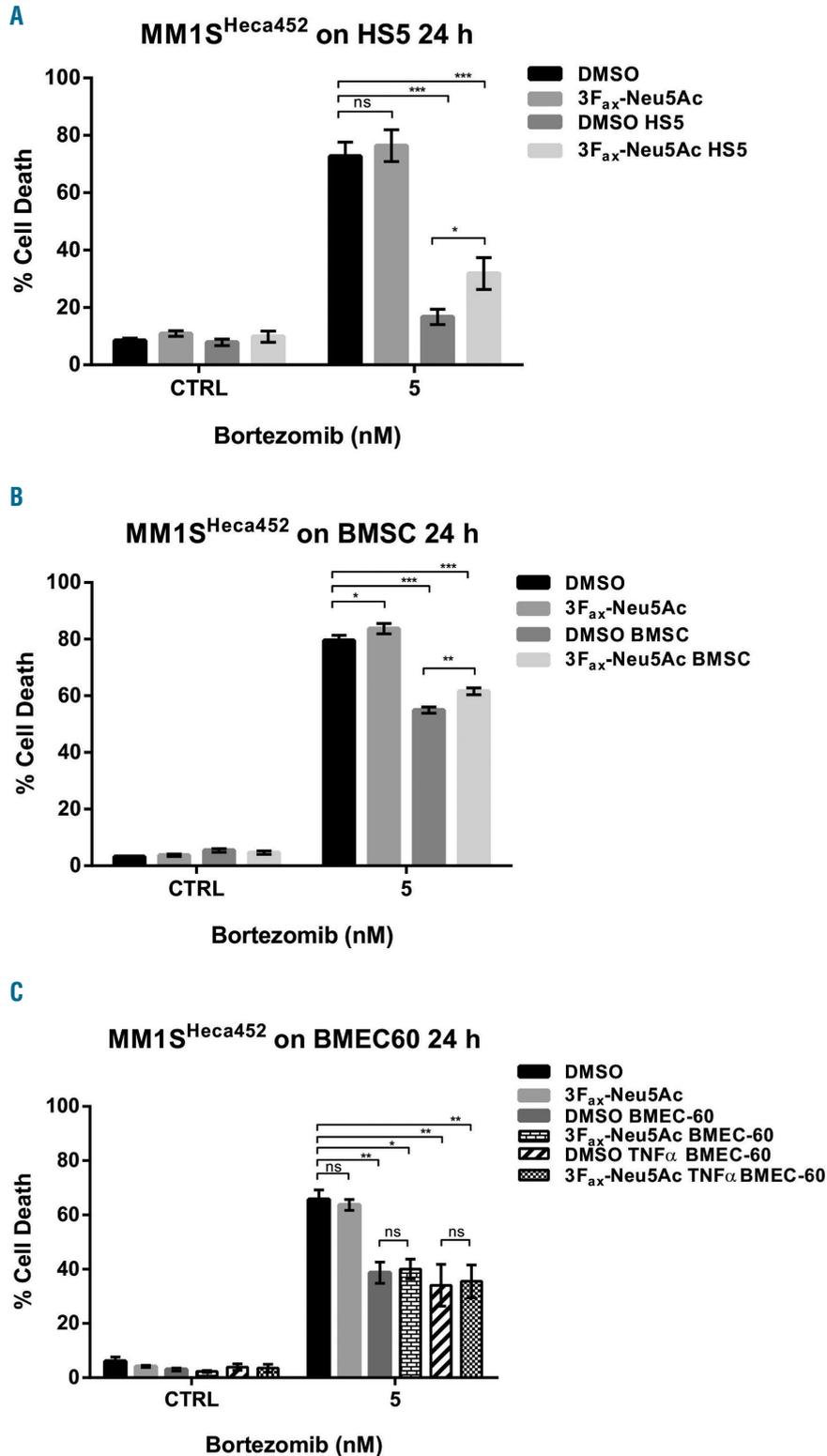
Figure 4. $3F_{ax}$-Neu5Ac treatment has a minimal impact on stroma-mediat- ed bortezomib resistance in MM1S[Heca452] cell line. MM1S[Heca452] cells were treated with 300 μM $3F_{ax}$-Neu5Ac or dimethyl sulfoxide (DMSO) [vehicle control (CTRL)] for seven days. After treatment, cells were seeded onto 80% confluent layer of HS5 expressing GFP (A), patient- derived bone marrow stromal cells (BMSC) (B) and BMEC-60 stained with Tag-it Violet™ (C) or plastic. BMEC-60 were also stimulated with 10 ng/mL TNFα for 4 hours (h) to induce activation. Cells were co-cul- tured for 24 h and then treated with 5 nM bortezomib for a further 24 h. After incubation, cells were collected and cell death was examined by Annexin V-APC and PI staining. One- way ANOVA was used to determine statistical significance with Dunnett's multiple comparison *post-hoc* testing. *P<0.05; **P<0.01; ***P<0.001; ns: non-significant.
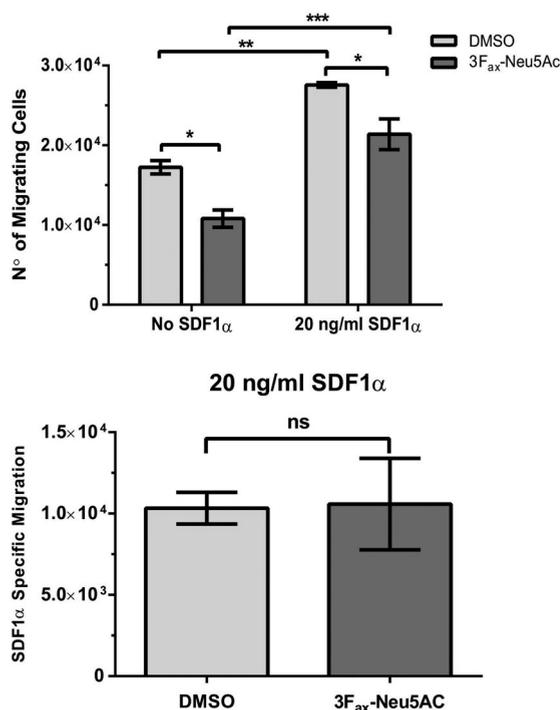
ical role of sialylation in melanoma metastasis and growth *in vivo*.[32]

The vehicle-pre-treated MM1S[Heca452] cells showed an initial response to bortezomib *in vivo*. Indeed, bortezomib was able to reduce tumor burden, however, despite this initial response, bortezomib alone was not able to improve survival. These data would suggest that the surviving MM1S[Heca452] cells were so aggressive that they still induced death in mice at a similar rate to the non-bortezomib-treated mice. $3F_{ax}$-Neu5Ac pre-treatment of MM1S[Heca452] cells effectively blocked α2-3 and α2-6 sialylation as well as expression of SLe[a/x]. More importantly, pretreatment of MM1S[Heca452] cells with $3F_{ax}$-Neu5Ac blunted

Figure 5. $3F_{ax}$-Neu5Ac treatment reduces motility of MM1S[Heca452] independently of SDF1α. MM1S[Heca452] cells were treated with 300 μM $3F_{ax}$-Neu5Ac or dimethyl sulfoxide (DMSO) (vehicle control) for seven days. After treatment, cells were starved for 1 hour (h) and then seeded on the upper chamber of transwells. Lower chamber was filled with either serum-free media (No SDF1α) or serum-free media supplemented with SDF1α (20 ng/mL). Cells were allowed to migrate for 4 h at 37°C. After incubation, cells in the lower chambers were collected and counted using a BD Accuri flow cytometer. Data are presented as (A) raw data or (B) as the difference between migrating cells in SDF1α-containing media and control media. Bars represents mean±Standard Error of Mean of three independent experiments. One-way ANOVA test was used to determine statistical significance with Sidak's multiple comparison *post-hoc* testing. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ns: non-significant.
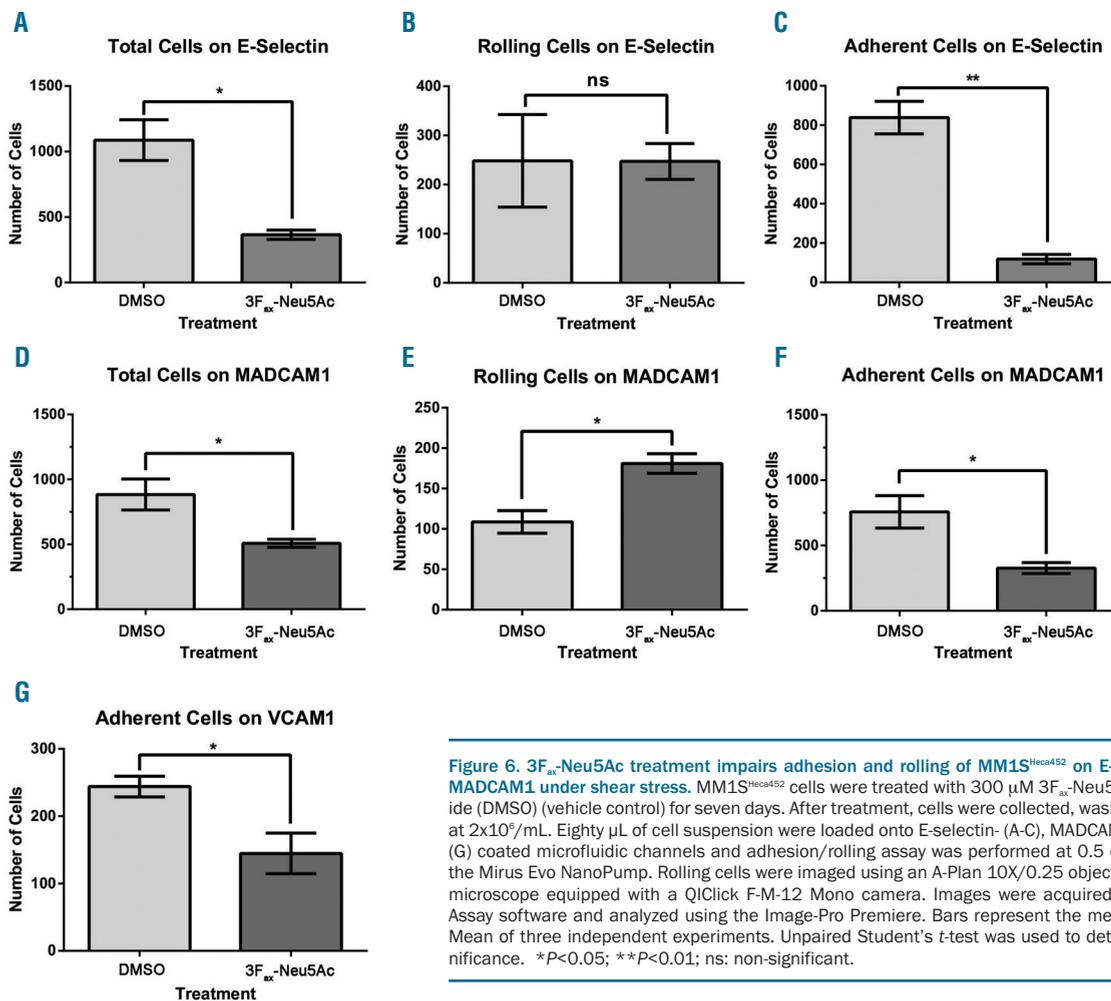

Figure 6. $3F_{ax}$-Neu5Ac treatment impairs adhesion and rolling of MM1S[Heca452] on E-selectin, VCAM1 and MADCAM1 under shear stress. MM1S[Heca452] cells were treated with 300 μM $3F_{ax}$-Neu5Ac or dimethyl sulfoxide (DMSO) (vehicle control) for seven days. After treatment, cells were collected, washed and resuspended at $2x10^6$/mL. Eighty μL of cell suspension were loaded onto E-selectin- (A-C), MADCAM1- (D-F) and VCAM1- (G) coated microfluidic channels and adhesion/rolling assay was performed at 0.5 dyne/cm^{2} at RT using the Mirus Evo NanoPump. Rolling cells were imaged using an A-Plan 10X/0.25 objective of an A10 Vert.A1 microscope equipped with a QIClick F-M-12 Mono camera. Images were acquired using the Vena Flux Assay software and analyzed using the Image-Pro Premiere. Bars represent the mean±Standard Error of Mean of three independent experiments. Unpaired Student's *t*-test was used to determine statistical significance. *$P<0.05$; **$P<0.01$; ns: non-significant.

the aggressive nature of these cells. Indeed, $3F_{ax}$-Neu5Ac treatment reduced tumor burden, increased bortezomib sensitivity and, most importantly, improved survival, suggesting that sialylation contributes to the aggressive phenotype of the MM1S[Heca452] cells and inhibiting it could represent a valuable treatment in MM. Currently, systemic administration of $3F_{ax}$-Neu5Ac is not feasible due to irreversible nephrotoxicity. While it is possible that a different dose and schedule could reveal a therapeutic window, clearly the potential for off-target toxicity is a major obstacle to clinical development. This is particularly relevant in MM where the kidney is one of the organs whose function is greatly impaired by the disease. However, alternative approaches could be explored to address this issue, including the development of more selective sialyltransferase inhibitors or the use of a targeted delivery system, which would release $3F_{ax}$-Neu5Ac selectively into the BM microenvironment or the MM cells. Indeed, Bull *et al.* have previously reported that targeted delivery of antibody-labeled nanoparticles containing $3F_{ax}$-Neu5Ac into melanoma cells facilitates long-term sialic acid blockade and, importantly, reduces lung metastasis *in vivo*.[33] A similar strategy could be employed using antibodies specific to MM antigens (such as CD38 and BCMA) or by incorporating bisphosphonates into the nanoparticles to target the BM.[34] Achievement of sufficiently high local BM concentrations of $3F_{ax}$-Neu5Ac should result in sialylation inhibition on MM cells, without off target toxicity. Inhibiting sialylation using these approaches could also target the tumor microenvironment including the immune environment. For instance, it has been recently reported that sialic acid blockade *via* intratumoral injection of $3F_{ax}$-Neu5Ac could suppress tumor growth by enhancing T-cell-mediated tumor immunity.[35] In addition to a reduction in sialic acid expression by tumor cells, sialyltransferase inhibition converted the immune suppressive tumor microenvironment to an immune promoting one with significantly higher numbers of activated effector immune cells, including CD8^{+} T cells and natural killer (NK) cells, along with a reduction in regulatory T cells (Tregs).[35] Sialyltransferase inhibition also

led to anti-tumor effects, which were mediated by CD8^{+} effector cells as well as potential activation of stimulated dendritic cells (DC).[35]

A number of different mechanisms could account for the $3F_{ax}$-Neu5Ac-mediated increased-sensitization of the MM1S[Heca452] cells to bortezomib *in vivo*. First, we explored the possibility that $3F_{ax}$-Neu5Ac could directly inhibit BM-mediated bortezomib resistance *in vitro*. HS5, patient-derived BMSC and the BM endothelial cell line BMEC-60 showed significant inhibition of bortezomib-induced cell death in MM1S[Heca452]. However, we observed that $3F_{ax}$-Neu5Ac induced only a partial re-sensitization to bortezomib on HS5 and patient-derived BMSC, suggesting that desialylation plays a minor role in blocking BM-mediated drug resistance. The BM microenvironment can induce drug resistance through cell adhesion-mediated drug resistance (CAM-DR) and soluble factors.[1,36-39] It is possible that in our *in vitro* model system, inhibition of sialylation is not enough to inactivate all the pathways responsible for the BM-mediated bortezomib resistance.[1,39,40] Therefore, it is conceivable that the increased-sensitization to bortezomib observed *in vivo* may be predominantly due to mechanisms other than blockade of BM-mediated drug resistance. Nonetheless, an important future direction will be to test $3F_{ax}$-Neu5Ac in *in vitro* models that more faithfully reproduce the tumor microenvironment to better understand the effects of $3F_{ax}$-Neu5Ac on the microenvironment-mediated drug resistance.

Previously, we showed that in the same model system, the small molecule glycomimetic GMI-1271, which inhibits interactions between E-selectin and E-selectin ligands, could increase the number of MM cell in circulation, where they are more susceptible to bortezomib.[14] In a similar way, we hypothesized that inhibition of sialylation by $3F_{ax}$-Neu5Ac could also inhibit homing and retention of MM cells in the BM. To this end, we examined the interaction of vehicle or $3F_{ax}$-Neu5A-treated MM1S[Heca452] with E-selectin under shear stress. We found that $3F_{ax}$-Neu5Ac treatment, by reducing SLe[a/x], effectively inhibited the interaction between the MM1S[Heca452] cells and E-selectin, confirming previous observations.[24] However, we rea-
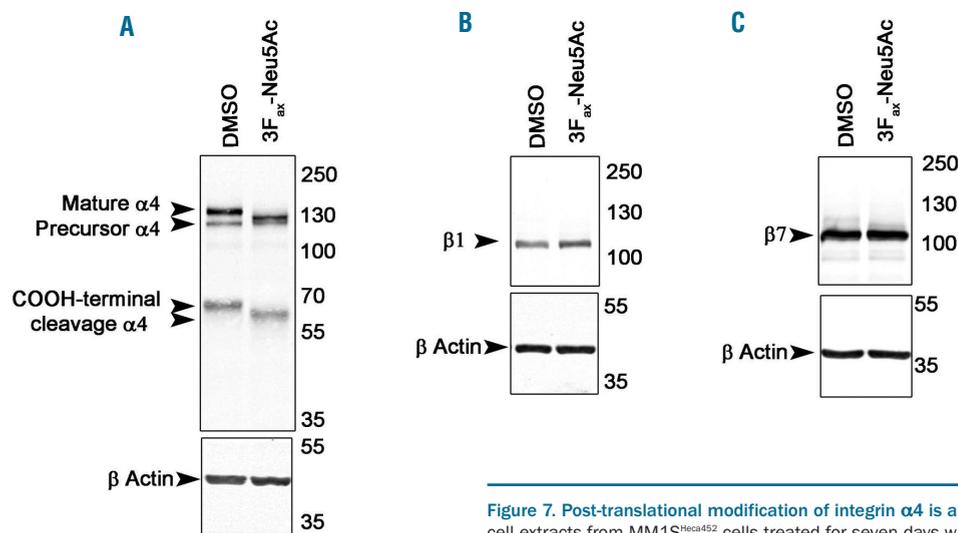
Figure 7. Post-translational modification of integrin α4 is altered by $3F_{ax}$-Neu5Ac treatment. Whole cell extracts from MM1S[Heca452] cells treated for seven days with 300 μM $3F_{ax}$-Neu5Ac or dimethyl sulfoxide (DMSO) (vehicle control) were subjected to SDS PAGE, transferred to nitrocellulose membrane and blotted for integrin α4 (A), β1 (B), and β7 (C).

soned that global suppression of sialylation could have effects beyond E-selectin. Indeed, $3F_{ax}$-Neu5Ac induced a general reduction in the motility of treated cells. This prompted us to investigate whether desialylation would alter adhesion and rolling mediated by α4β7 and α4β1 integrins, which are highly expressed on MM cells.[41-44] In a shear stress adhesion assay, we observed that $3F_{ax}$-Neu5Ac reduced the number of adherent cells on VCAM1 and, surprisingly, adhesion on MADCAM1. MADCAM1 is an immunoglobulin superfamily adhesion molecule expressed by mucosal venules that helps direct lymphocyte trafficking into Peyer's patches and the intestinal lamina propria.[29,45] There is also evidence that interaction between HSC and endothelial MADCAM1 in the BM promotes the homing and engraftment of HSC in mice.[46-48] In a similar way, MADCAM1 could co-operate with SDF1α and E-selectin to facilitate homing of MM cell in the BM. Indeed, MADCAM1 ligand α4/β7 has been shown to play a critical role in MM-cell adhesion, migration, invasion, BM homing, and adhesion-mediated drug resistance.[43,49] Moreover, it was shown that the expression levels of β7 integrin on MM cells correlates with poor survival in MM patients.[43] Our results suggest the possibility of reduced interactions between endothelial MADCAM1 and α4/β7 on MM cells as a result of desialylation. Indeed, we showed that $3F_{ax}$-Neu5Ac altered the SDS-PAGE mobility of the α4 chain and in particular of its mature forms, suggesting that desialylation interferes with α4 maturation. The interaction between MM cells and MADCAM1 becomes apparent only under shear stress as we failed to detect adhesion on MADCAM1 under static conditions (*data not shown*). This is highly reminiscent of L-selectin on leukocytes that requires a threshold shear stress to establish rolling and adhesion, below which no interactions are observed.[50] Thus, it is possible that MADCAM1 mediates or facilitates homing but not retention of the MM cells in the BM.

In conclusion, targeting sialylation in MM cells has the potential to block the ability of MM cells to home to the BM, which, in turn, could reduce the severity of the disease, because most existing therapies against MM, like bortezomib, are maximally effective on circulating MM cells.

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