

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

Alessandro Natoni,^{1*} Mariah L. Farrell,^{2,3,4*} Sophie Harris,^{2,3,4}
Carolyn Falank,^{2,3,4} Lucy Kirkham-McCarthy,¹ Matthew S. Macauley,⁵
Michaela R. Reagan^{2,3,4**} and Michael O'Dwyer^{1**}

¹Apoptosis Research Center, National University of Ireland, Galway, Ireland; ²Maine Medical Center Research Institute, Scarborough, ME, USA; ³Tufts University School of Medicine, Boston, MA, USA; ⁴Graduate School of Biomedical Science and Engineering, University of Maine, Orono, ME, USA and ⁵Department of Chemistry and Department of Medical Microbiology and Immunology, University of Alberta, Alberta, Canada

*AN and MLF contributed equally to this work. **MRR and MOD are joint senior authors of this work.

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Correspondence: MICHAEL O'DWYER - michael.odwyer@nuigalway.ie

Supplemental Methods

Retro-orbital Bleeds and Red Blood Cell Lysis. After mice were anesthetized with isoflurane, 100 μ l of blood was collected with a heparinized capillary tube from the medial canthus of the eye under the nictitating membrane. Slight pressure with a piece of gauze was applied to the eyeball to prevent further bleeding. Alternate eyes were used for each day of blood collection. Red blood cells (RBCs) were lysed for 10 minutes with 900 μ l red blood cell lysis buffer per 50 μ l of blood. RBC buffer contained 80.2 g NH_4Cl (Sigma Aldrich; St. Louis, MO), 11.3 mM HKCO_3 (Sigma Aldrich), 3.7 gm EDTA (Sigma Aldrich), topped to 1 liter with Millipore water. The white blood cells were then resuspended in 1 mL of PBS (VWR; Radnor, PA) reading for flow cytometry staining.

Bone Marrow Isolation. After mice were sacrificed with isoflurane, the right tibia and femur were harvested and cleaned with a kimwipe. The bone marrow was then flushed into a 70 mm filter with a 20 gauge needle using 1 unit/mL heparinized PBS. The bone marrow was then pushed through the filter. The filter was then rinsed with 5 mL of chilled PBS and all of the single cell suspension was collected and stored on ice.

Spleen isolation. After mice were sacrificed with isoflurane the spleen was harvested, cleaned and cut into small segments inside a 70 mm filter. The spleen segments were then pushed through the filter, which was subsequently rinsed with chilled PBS. The splenocytes were collected, and after centrifugation the supernatant was discarded. 1 mL of 1X ACK (Lonza, Walkersville, MD) lysis buffer was added for 5 minutes for RBCs lysis. The lysis was stopped with 10x volume of PBS.

Histology and Lectin Histochemistry. Carnoy's solution containing ethanol (VWR), chloroform (Amresco; Solon, OH) and acetic acid (Sigma Aldrich) was used to fix paraffin embedded spleen, liver and kidney samples processed for lectin histochemistry. Endogenous peroxidase was blocked by treating tissues with 3% hydrogen peroxide in methanol for 30 minutes, and then incubated in 10 mM citrate buffer, pH 6.0, for 40 min at 85 °C. Sections were blocked in Hanks buffered salt solution that contained 5% Bovine Serum Albumin (BSA, Sigma Aldrich) and 2 mM Calcium dichloride for 30 min and rocked overnight at 4°C with biotinylated *Sambucus Nigra* (SNA; spleen, 1:3,000; liver, 1:10,000; kidney, 1:1,000) or biotinylated *Peanut Agglutinin* (PNA, 1:10,000; all from Vector Laboratories; Burlingame, CA). Formalin-fixed paraffin embedded tissue sections of kidney samples were processed with hematoxylin and eosin (H&E) staining.

Cell lines and culture conditions. The MM cell lines GFP⁺/Luc⁺ MM1S and parental MM1S were sourced from Dr. Irene Ghobrial (Dana-Farber Cancer Institute, Boston, MA) and American Type Culture Collection (ATCC; Manassas, VA) respectively. The Heca452 variant for both cell lines was generated by two round of cell sorting using the Heca452 antibody. Cells were maintained in RPMI-1640 (VWR) containing L-glutamine, 10% heat inactivated fetal bovine serum (HI-FBS, VWR), 1X antibiotic-antimycotic (Corning; Kennebunk, ME). The HS5

cell line was from ATCC. The GFP⁺ variant was generated by lentiviral transduction of the HS5 with GFP-expressing construct and subsequent sorting of GFP⁺ cells. The cells were maintained in DMEM supplemented with 10% HI-FBS, 50 U/ml penicillin (Sigma-Aldrich), and 50 µg/ml streptomycin (Sigma-Aldrich). The BMEC-60 cells were from Prof. Ellen C. van der Schoot (Academic Medical Center, University of Amsterdam, The Netherlands) and were maintained in EGM-2MV medium (Lonza; Basel, Switzerland). Patient-derived BMSC were isolated from MM bone marrow samples by plastic adherence and cultured in αMEM (Sigma-Aldrich) supplemented with 10% HI-FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 1% sodium pyruvate and 1% non-essential amino-acids (all from Sigma-Aldrich). Bone marrow samples from MM patients were obtained with informed consent and ethical approval of the local Ethics Committee in accordance with the Declaration of Helsinki.

Fluorescence-activated cell sorting. In toxicity study, cells extracted from mice were stained with anti-CD19, anti-CD3, anti-CD45, anti-Ly6C, anti-CD11b (all from Biolegend, San Diego, CA), biotinylated SNA, PNA or *Maackia Amurensis* Lectin II (MALII, all from Vector Laboratories) lectins and subsequently detected by FITC-streptavidin (Biolegend) in FACS buffer (0.5% BSA and 2mM EDTA). Sialic acid on the surface of the MM1S^{Heca452} and parental cell line was detected using biotinylated SNA and MALII (all 1:10,000 in FACS buffer, 30 min incubation at RT) followed by 15 min incubation at RT with APC-streptavidin beads (BD Biosciences; San Jose, CA). To detect sialofucosylated structures responsible for E-selectin binding, cells were stained with the PE-Heca452 and BV421-CD15s antibodies (both from BD Biosciences) for 30 min at RT. To detect surface expression of integrins, cells were stained with PE-anti α4 (clone 9F10), β1 (clone MAR4) and β7 (clone FIB504; all from BD Biosciences) and incubated as above. Before acquisition, cells were stained with 1 µM 7-Aminoactinomycin D (7-AAD, Thermo Scientific; Waltham, MA) to discriminate between live and dead cells. Data was collected using either FACS Canto II (BD Biosciences) or MACS Quant (Miltenyi Biotec; Bergisch Gladbach, Germany) flow cytometer and analyzed using FlowJo V10 (FlowJo LLC; Ashland, OR) or Infinicyt 2.0 (Cytognos; Salamanca, Spain).

In vitro cytotoxicity assay. HS5 GFP⁺, patient-derived BMSC and BMEC cells were culture at approximately 80% confluency. To induce activation, BMEC-60 cells were also stimulated with 10 ng/ml TNFα (PeProtech; Rocky Hill, NJ) for 4 h before MM seeding. 3F_{ax}-Neu5Ac or DMSO (vehicle control) treated MM1S^{Heca452} (2.5x10⁵ cells/ml) cells were seeded (0.5 ml) on HS5 GFP⁺, patient-derived BMSC or BMEC-60 cells and incubated for 24 h in a humidified incubator at 37°C and 5% CO₂. Cells were then treated with 5 nM Bortezomib (Selleck; Houston, TX) for further 24 h. After incubation, cells were collected and stained with APC conjugated Annexin V (ImmunoTools; Friesoythe, Germany) and Propidium Iodide (PI, Sigma-Aldrich). Cells were acquired using a FACS CANTO II and cell death was analyzed using Infinicyt. HS5 GFP⁺ cells were discriminated from the MM cells using the 530/30 nm channel. To discriminate patient-derived BMSC and BMEC-60 from the MM cells, patient-derived BMSC and BMEC-60 cells were stained with 2.5 µM Tag-it Violet™ Proliferation and Cell Tracking Dye (Biolegend) for 15 min at RT in the dark. After incubation, excessive dye was quenched by adding DMEM for 10

min. After incubation, patient-derived BMSC and BMEC-60 cells were washed and resuspended in growth media at the desired volume. Patient-derived BMSC and BMEC-60 were discriminated from the MM cells using the 450/50 nm channel.

Migration Assay. 3F_{ax}-Neu5Ac or DMSO (vehicle control) treated MM1S^{Heca452} cells were starved for 1 hour and then seeded (2.5×10^5 cells in 200 μ l) on the top chamber of a transwell (poly-carbonate membrane, 8 μ m pore size, Corning). The lower chamber was filled with 600 μ l serum free RPMI-1640 media with/without 20 ng/ml SDF1 α (PeProtech). Cells were incubated for 5 hours, migrated cells were collected from the bottom chamber and counted using a BD Accuri C6 flow cytometer.

Rolling and adhesion assay under shear stress. Eight channel microfluidic biochips (Cellix Limited; Dublin, Ireland) were coated with 15 μ g/ml of E-selectin (PeProtech), 15 μ g/ml VCAM and MADCAM1 chimeras (R&D System; Minneapolis, MN) in Tris-HCl pH 7.4 supplemented with 1 mM CaCl₂ (coating buffer) and incubated overnight at 4°C. Each channel was blocked with 1% BSA and incubated at 37°C 1 h before the assay. 3F_{ax}-Neu5Ac or DMSO (vehicle control) treated MM1S^{Heca452} cells were washed and resuspended in assay buffer (RPMI-1640 media without phenol red supplemented with 1% HI FBS, 5 mM Hepes and 1 mM CaCl₂) at 2.5×10^6 cells/ml. Eighty μ l of cell suspension were loaded onto the microchannels and the assay was run at 0.5 dyne/cm² at RT using a Mirus Evo Nano Pump (Cellix Limited). Cells were monitored in 5 different positions along the channel using an A-Plan 10X/0.25 objective (Carl Zeiss Microscopy GmbH; Jena, Germany) of an AX10Vert.A1 Microscope (Carl Zeiss). Thirty frames per position were collected at 0.5 sec from each other using a 01 QIClick F-M-12 Mono 12-bit camera (QImaging; Surrey, Canada). Images were acquired using the Vena Flux assay software (Cellix Limited) and the analysis was performed using the Image-Pro Premiere software (Media Cybernetics; Rockville, MD). A rolling cell was defined as a cell travelling a distance corresponding to more than its diameter. The number of cells per position were added to obtain the total number of cells per channel which was then averaged between the numbers of channel. Cells did not adhere nor roll on microchannels coated with Fc chimera or 1% BSA (data not shown).

Western Blot analysis. Cells (5×10^6) were lysed in 2% NP-40 buffer (150 mM NaCl, 50 mM Tris•HCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (1X, Thermo Scientific). Protein concentration was assessed by Bicinchoninic acid (BCA, Thermo Scientific). 20 μ g of protein was resolved on 8% SDS PAGE, transferred to nitrocellulose membrane and blocked for 1 h with 5% not fat milk proteins in PBS. Blots were probed overnight with α 4 (EPR1355Y), β 1 (EPR16896) and β 7 (EPR1357) (all from Abcam; Cambridge, UK) diluted 1:1000 in 5% BSA in PBS containing 0.05% Tween (V/V). Primary antibodies were detected with infrared conjugated goat anti-rabbit secondary antibodies (Li-cor; Lincoln, NE) diluted 1:10,000 in 5% milk in PBS containing 0.05% Tween. Blots were imaged using a Li-cor Odyssey system.

Supplemental Figure 1. Systemic decrease of sialylation monitored by SNA staining following 3F_{ax}-Neu5Ac treatment. Representative images of SNA lectin histochemistry on kidney (A), spleen (B) and liver (C) tissues in either vehicle or 3F_{ax}-Neu5Ac treated mice (6.25, 12.5 and 25 mg/Kg). Images were taken after seven days of treatment at 20X magnification of 4 representative mice.

Supplemental Figure 2. Systemic decrease of sialylation monitored by PNA staining following 3F_{ax}-Neu5Ac treatment. Representative images of PNA lectin histochemistry on kidney (A), spleen (B) and liver (C) tissues in either vehicle or 3F_{ax}-Neu5Ac treated mice (6.25, 12.5 and 25 mg/Kg). Images were taken after seven days of treatment at 20X magnification of 4 representative mice.

Supplemental Figure 3. Time-dependent decrease of the Heca452 marker in MM1S^{Heca452} following treatment with 3F_{ax}-Neu5Ac. MM1S^{Heca452} cells were treated with 300 μM 3F_{ax}-Neu5Ac, stained at the indicated time points using Heca452 antibody and analyzed by flow cytometry. Changes in (A) the median fluorescence intensity (MFI) and (B) the number of Heca452-positive cells are reported. Bars represents mean ± SEM of three independent experiments. The unpaired Student's T-test was used to determine statistical significance between DMSO and 3F_{ax}-Neu5Ac-treated cells for each individual time point. *** p<0.001; ** p<0.01; ns: non-significant.

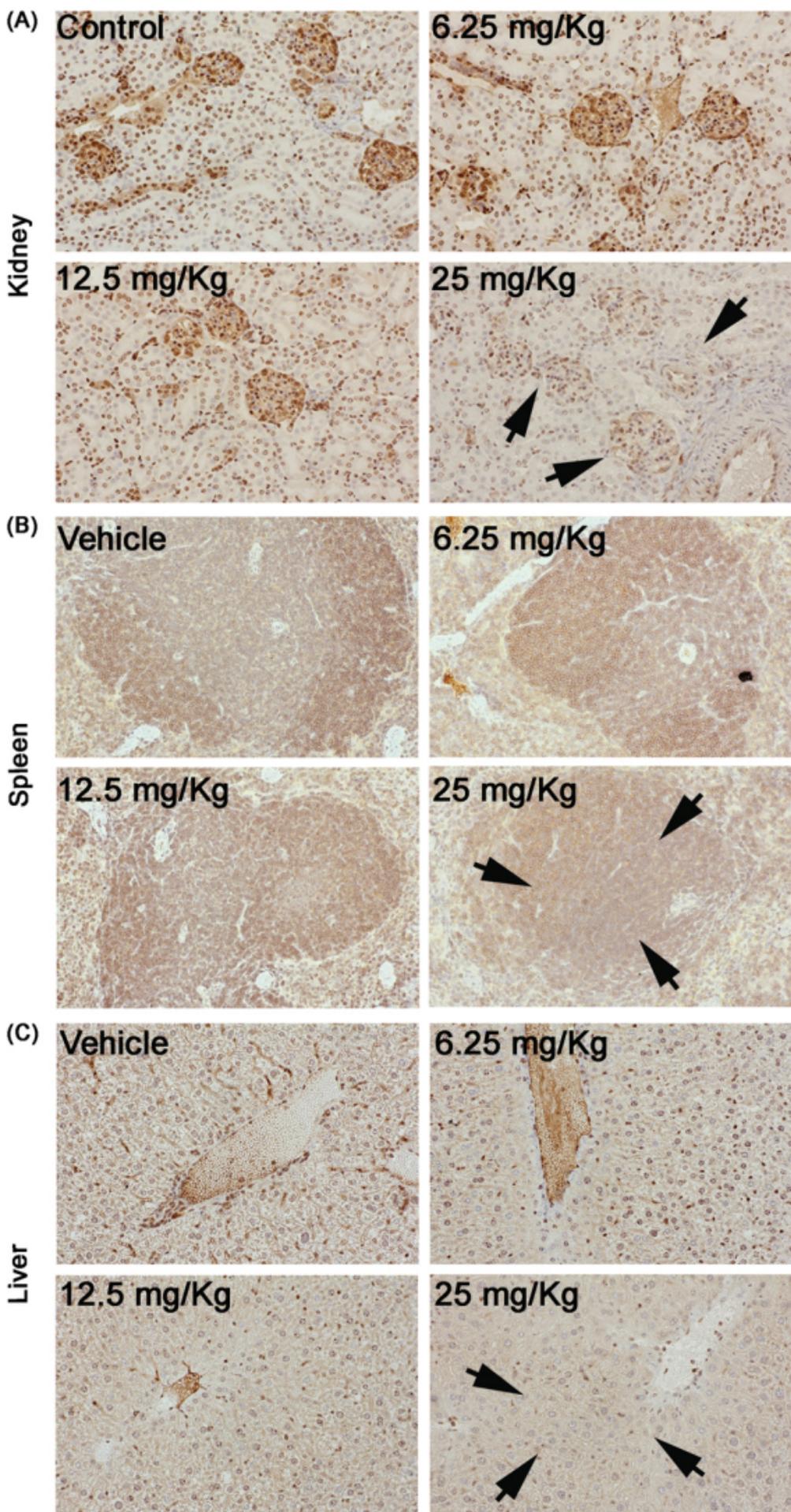
Supplemental Figure 4. 3F_{ax}-Neu5Ac treatment increases the velocity of the rolling fraction on E-selectin and MADCAM1. Rolling velocity of the MM1S^{Heca452} cells treated with 300 μM 3F_{ax}-Neu5Ac or DMSO (vehicle control) on E-selectin (A-C) and MADCAM1-coated channels (D-F). Each dots represent the velocity of a single cell. The three independent experiments for E-selectin and MADCAM1 are shown. Bars represent median ± interquartile range. The non-parametric Mann-Whitney test was used to determine statistical significance. *** p<0.001; ** p<0.01.

Supplemental Figure 5. 3F_{ax}-Neu5Ac treatment does not modulate Integrin α4, β1 and β7 expression. MM1S^{Heca452} cells were treated with 300 μM 3F_{ax}-Neu5Ac or DMSO (vehicle control) for 7 days. After treatment, cells were collected and stained with Integrin α4 (A, B), β1 (C, D) and β7 (E, F). Bars represent mean ± SEM of three independent experiments. The unpaired Student's T-test was used to determine statistical significance. ns: non-significant.

Supplemental Figure 6. Post-translational modification of Integrin α4 is altered by 3F_{ax}-Neu5Ac treatment in both MM1S parental and MM1S^{Heca452} cells. Whole cell extracts from parental and MM1S^{Heca452} cells treated for 7 days with 300 μM 3F_{ax}-Neu5Ac or DMSO (vehicle control) were subjected to SDS PAGE, transferred to nitrocellulose membrane and blotted for integrin α4 (A), β1 (B) and β7 (C).

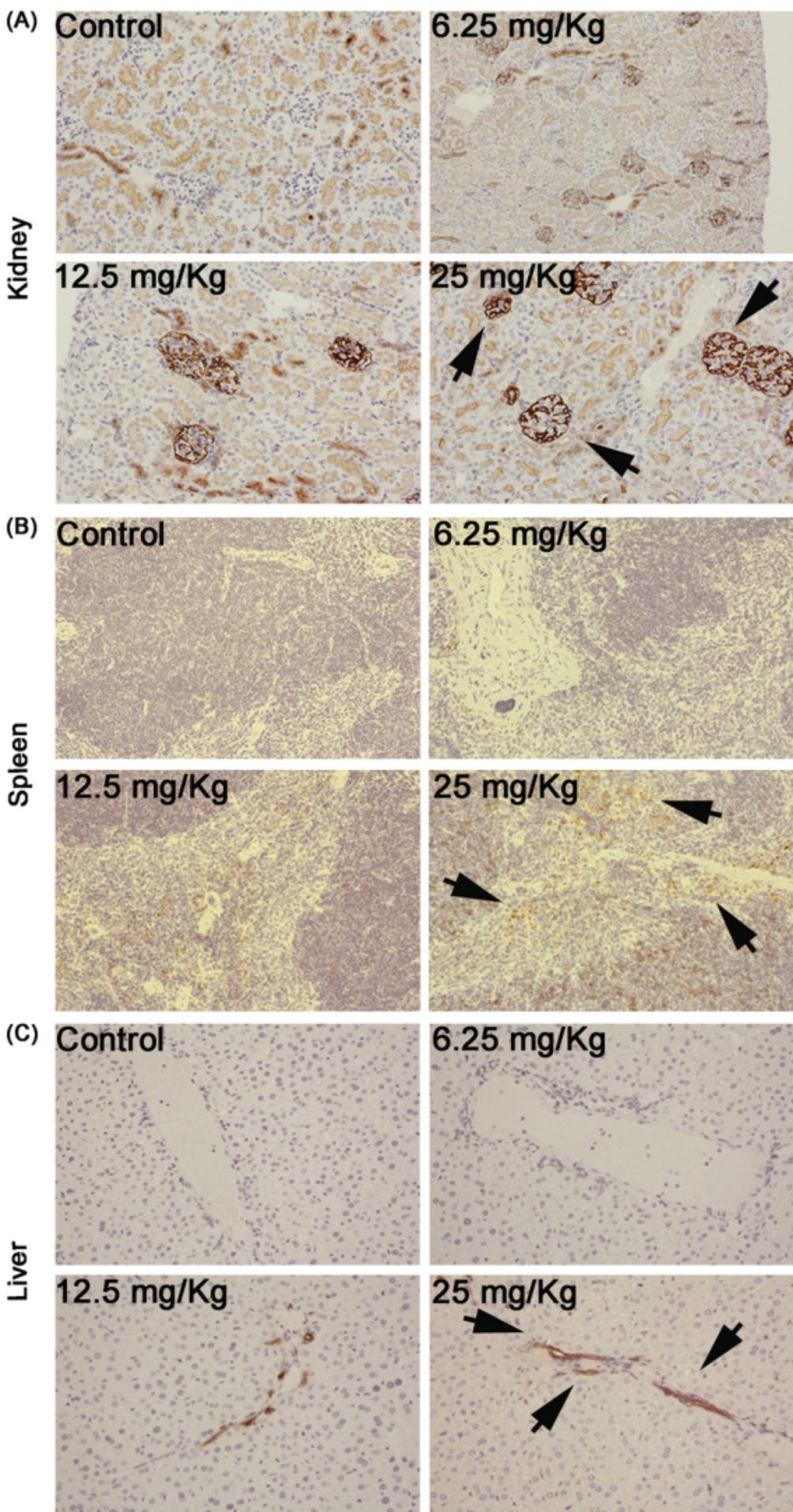
Supplemental Figure 1

SNA Staining

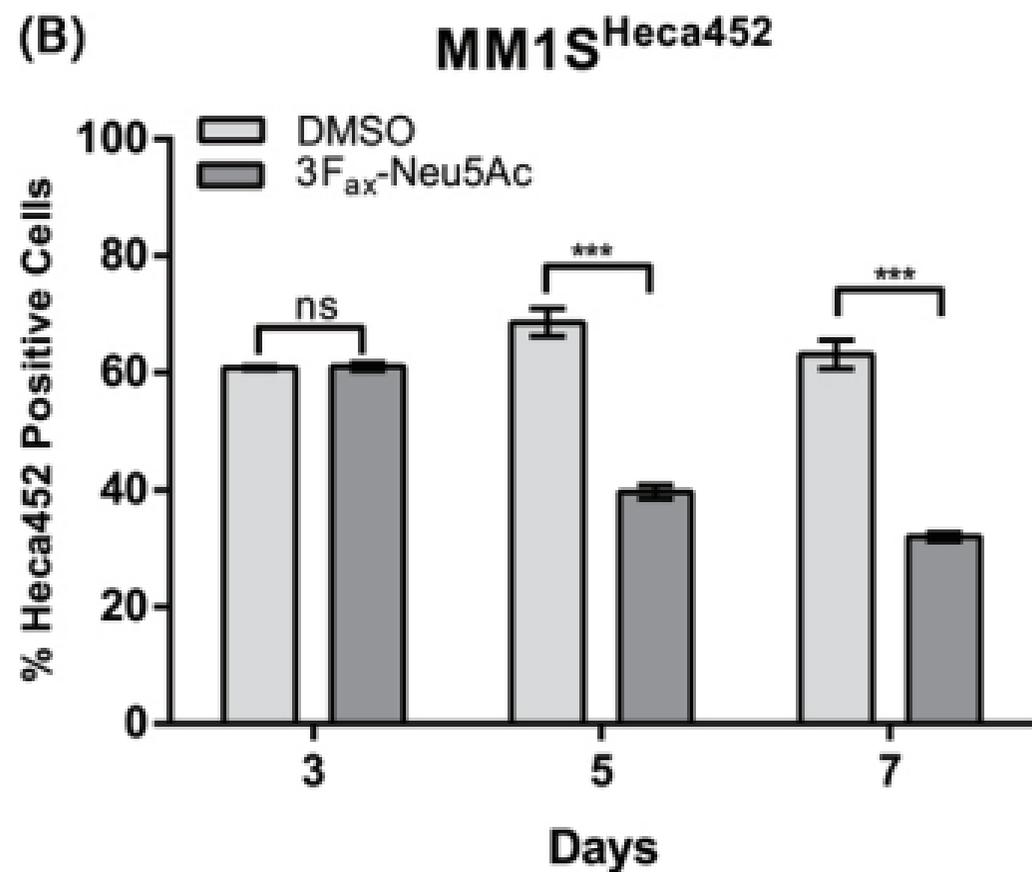
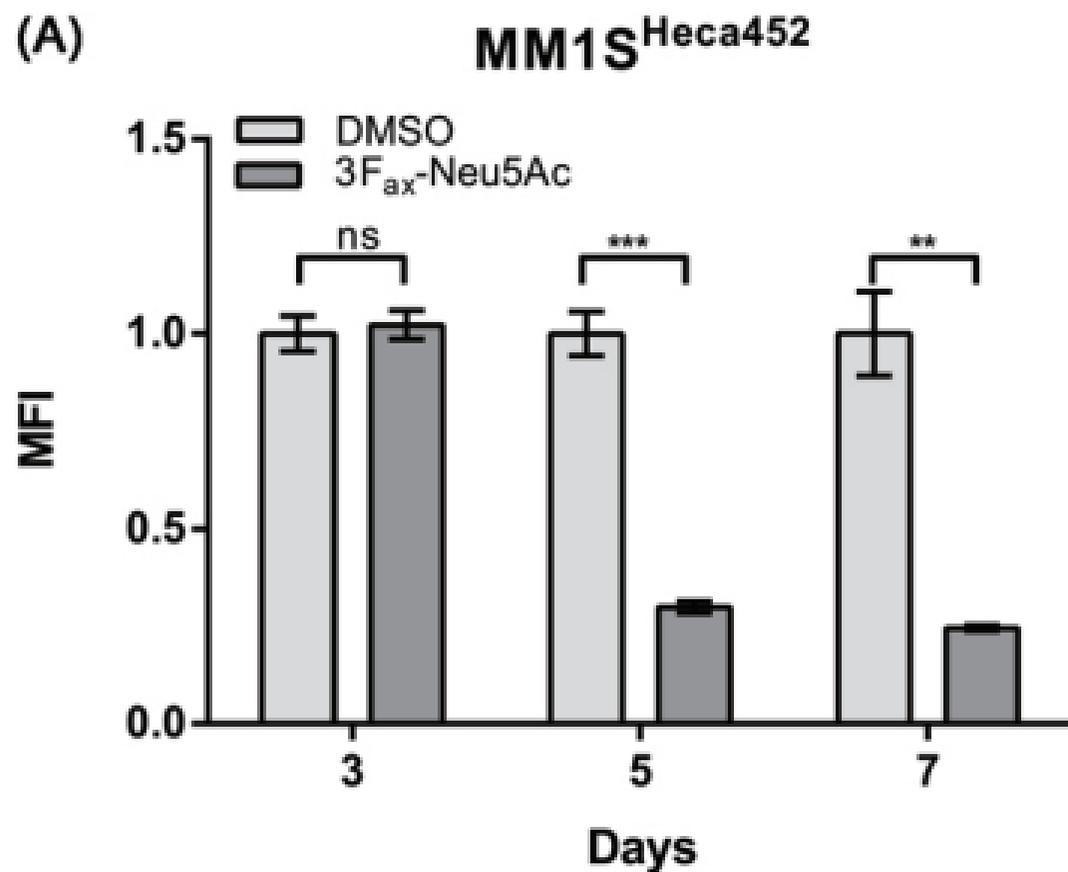


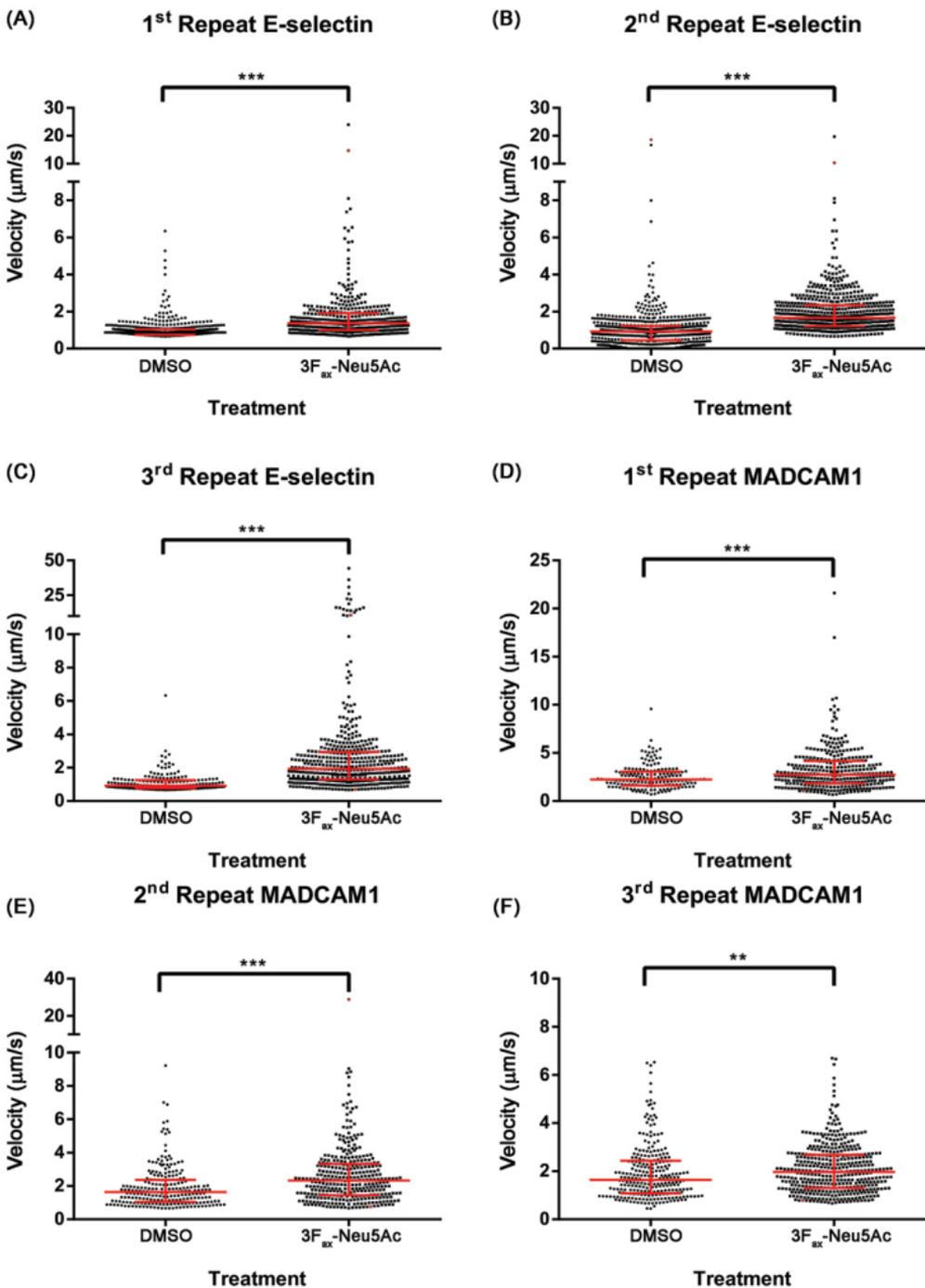
Supplemental Figure 2

PNA Staining

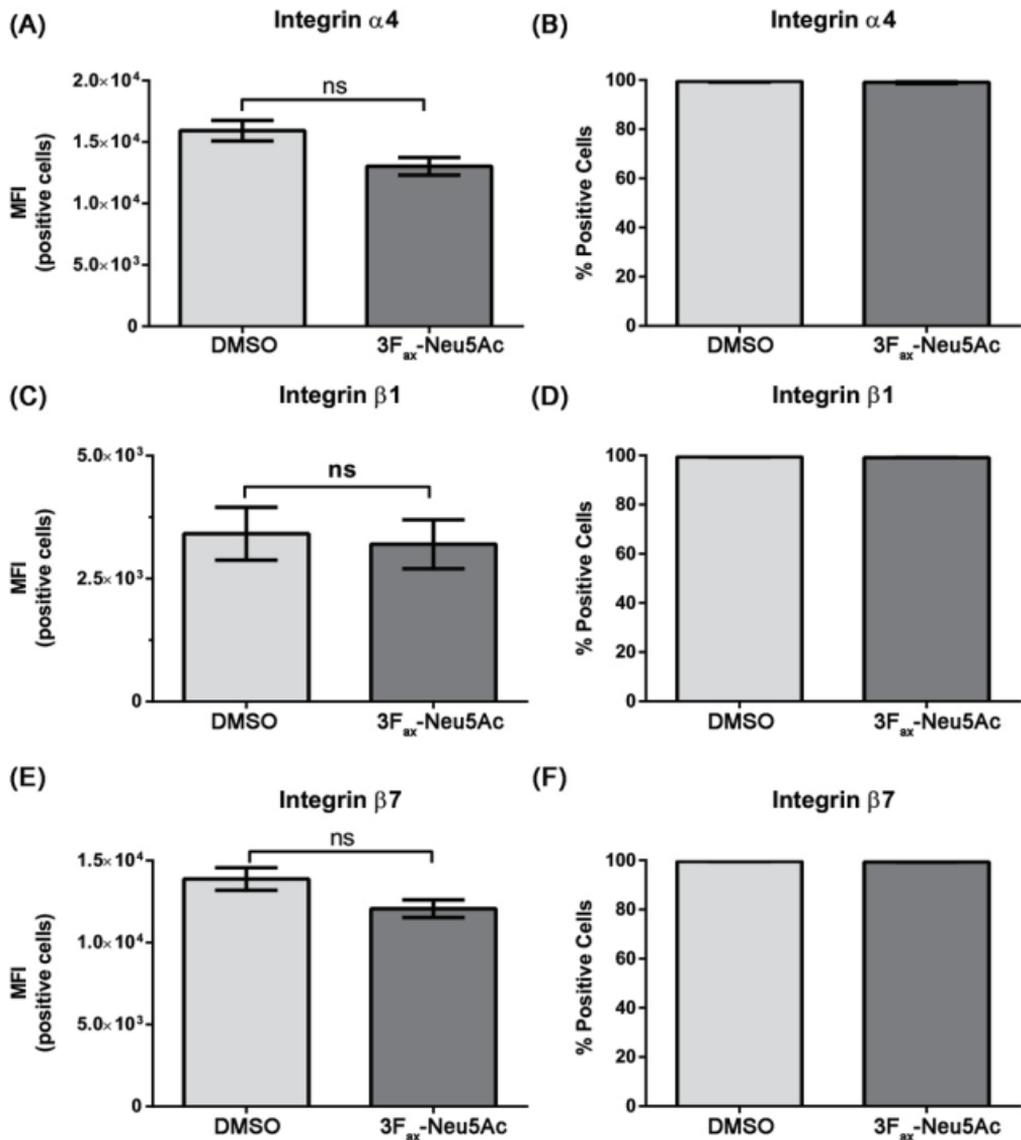


Supplemental Figure 3



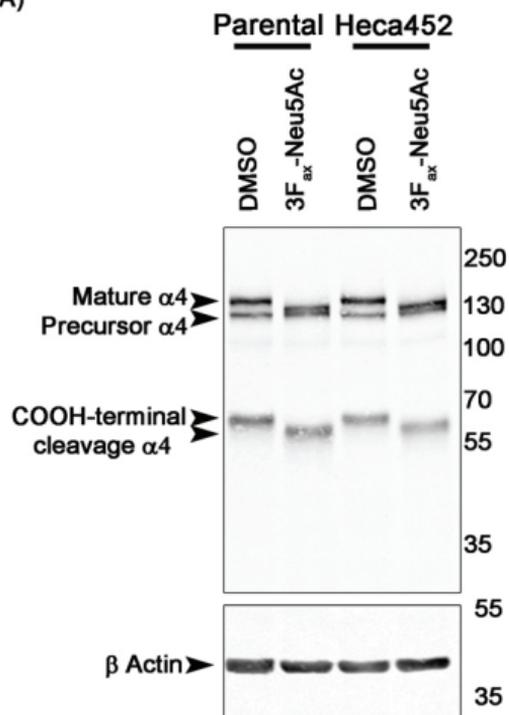


Supplemental Figure 5

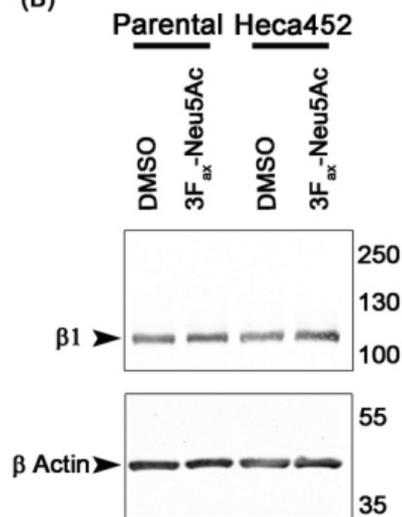


Supplemental Figure 6

(A)



(B)



(C)

