Self-assembling Multidomain Peptide Hydrogels accelerate Peripheral Nerve Regeneration after Crush Injury

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Materials and Methods:

Peptide synthesis: All amino acids and synthesis reagents were purchased from EMD Chemicals (Philadelphia, PA), solvents, diisopropylethylamine (DiEA), scavengers, and Trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). The peptides were synthesized using a standard FMOC based solid-phase peptide synthesis manually for small scale or on an Apex Focus XC (AAPPTec) automatic synthesizer for a larger scale. The scales of the synthesis were 0.15 for K_2-IIKDI, K_2-IKVAV, K_2-TenC, K_2-KDI, or 0.45 mmol for the rest of the peptides. A Low-loading Rink Amide MBHA resin (0.32-0.36 mmol/g, EMD Millipore) was used to have an amidated C-terminus. All reagents were dissolved in DMF-DMSO (1:1). For each amino acid coupling cycle, 4 equivalents of amino acid, 4 equivalents of HATU, and 6 equivalents of DiEA were used. The reaction mixture was shaken for at least 1 min for activation and added to the deprotected resin or peptide. Coupling cycles were 25 min and 45 min in manual or automatic synthesis, respectively. Deprotection of the Fmoc protecting group was achieved with two treatments with 20% (v/v) piperidine in DMF:DMSO for 7 min each. Effective coupling or deprotection reactions were tested using standard ninhydrin or chloranil tests. After the peptide sequence was completed, the N-terminus was acetylated by acetic anhydride and DiEA in DMF twice for 45 min each. Then, the peptide was cleaved from the resin with a TFA/triisopropylsilane/anisole/ethanedithiol/H_2O (18:0.5:0.5:0.5:0.5 by volume) cleavage cocktail for 3 hours at room temperature. Excess TFA was evaporated, and the peptides were obtained by trituration with cold diethyl ether. The peptide powder was collected by centrifugation, rinsed 3 times with cold diethyl ether and allowed to dry overnight before dialysis and processing.

Transmission Electron Microscopy (TEM): Peptide solutions of 0.01% or 0.02% by weight in Milli-Q water were spotted on Quantofoil R1.2/1.3 holey carbon films on copper mesh grids. Samples were allowed to absorb for 1 min before blotting the excess. Negative staining was performed for 5 min using 2% by weight Phosphotungstic acid (PTA) solution at pH 7. Samples were air-dried and imaged at 120kV using a JEOL 2010 TEM microscope (JEOL Inc., Peabody, MA.).

Blinding procedure:

In vitro study: Peptide solutions were prepared and labeled with a single-letter code by one experimentalist. Cell seeding, culture, staining, and data analysis were performed by a blinded experimentalist. The identity of each sample was revealed after sholl analysis.

In vivo study: After hydrogel preparation, each material was assigned a letter code and their identity hidden for the surgeon and experimentalist. Animals were assigned a number and ear-tagged before surgery. Surgeries, tissue harvesting, tissue analysis, and walking track were performed blinded using the animal ID solely. The identity of each material was revealed after data analysis.

Table S1. Peptide sequences of self-assembling Multidomain Peptides containing bioactive peptide mimics

Name	Peptide sequence	Monoisotopic mass (Da)	Molecular weight (Da)	Hydrogel concentration
K_2*	$K_2(SL)_6K_2$	1772.10	1773.17	5.6 mM
O_5*	$(Hyp)_5(SL)_6(Hyp)_5$	2390.25	2391.64	4.2 mM
SLac*	$K(SL)_3RG(SL)_3KGRGDS$	2201.24	2202.52	4.5 mM
SLanc*	$K(SL)_3RG(SL)_3KGKLTWQELYQLKYKGI$	3678.11	3680.37	2.7 mM
K_2-IIKDI	$K_2(SL)_6K_2GRNIAEIIKDI$	2994.80	2996.07	6.6 mM
K_2-IKVAV	$K_2(SL)_6K_2GIKVAV$	2339.48	2340.90	4.3 mM
K_2-TenC	$K_2(SL)_6K_2GVFDNFVLK$	2791.65	2793.36	3.5 mM
K_2-KDI	$K_2(SL)_6K_2GKDI$	2185.33	2186.44	4.5 mM

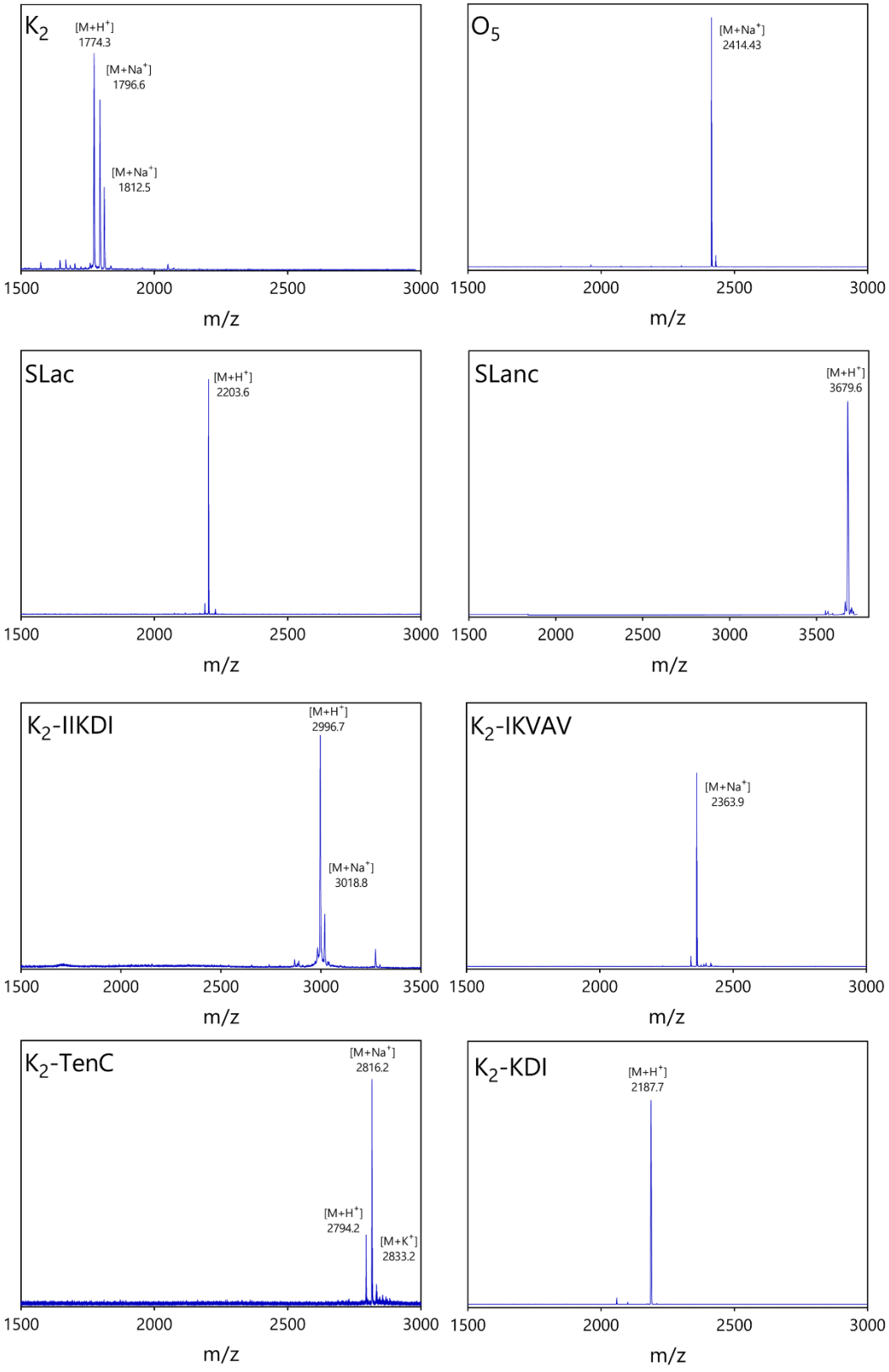

Figure S1. MALDI-TOF MS spectra of multidomain peptides.

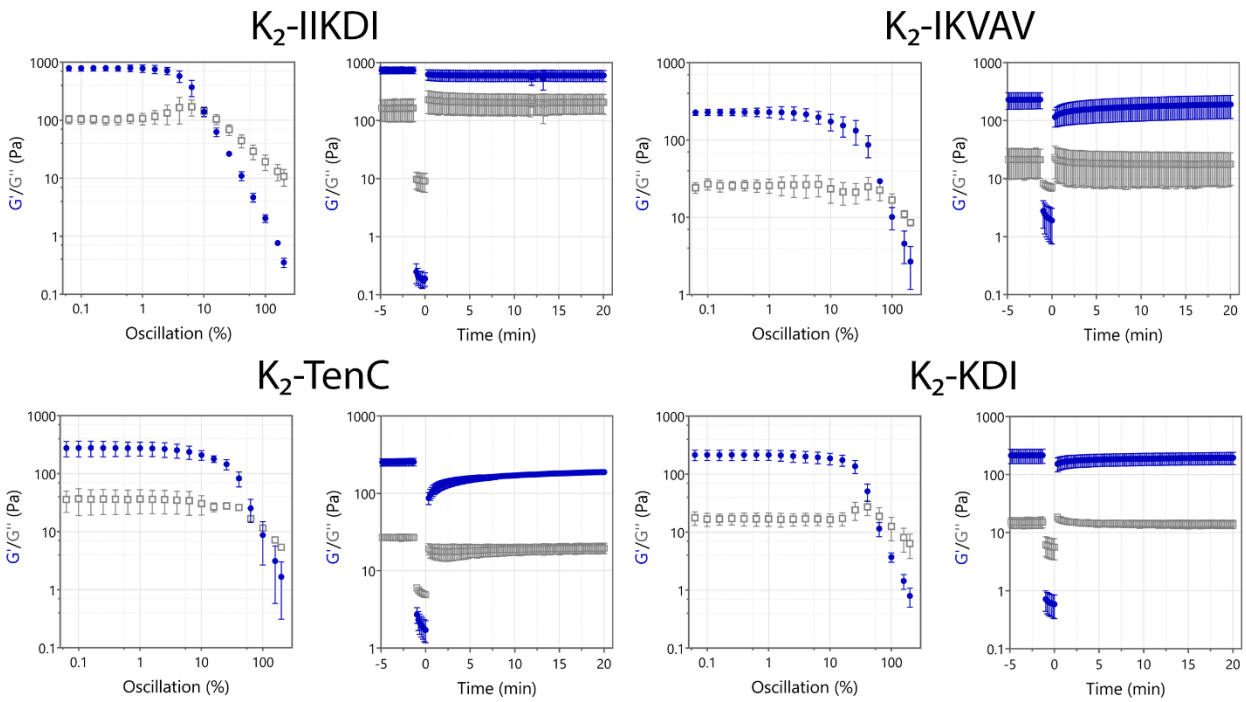

Figure S2. Oscillatory Rheology of functionalized K_2 MDPs (n = 3).

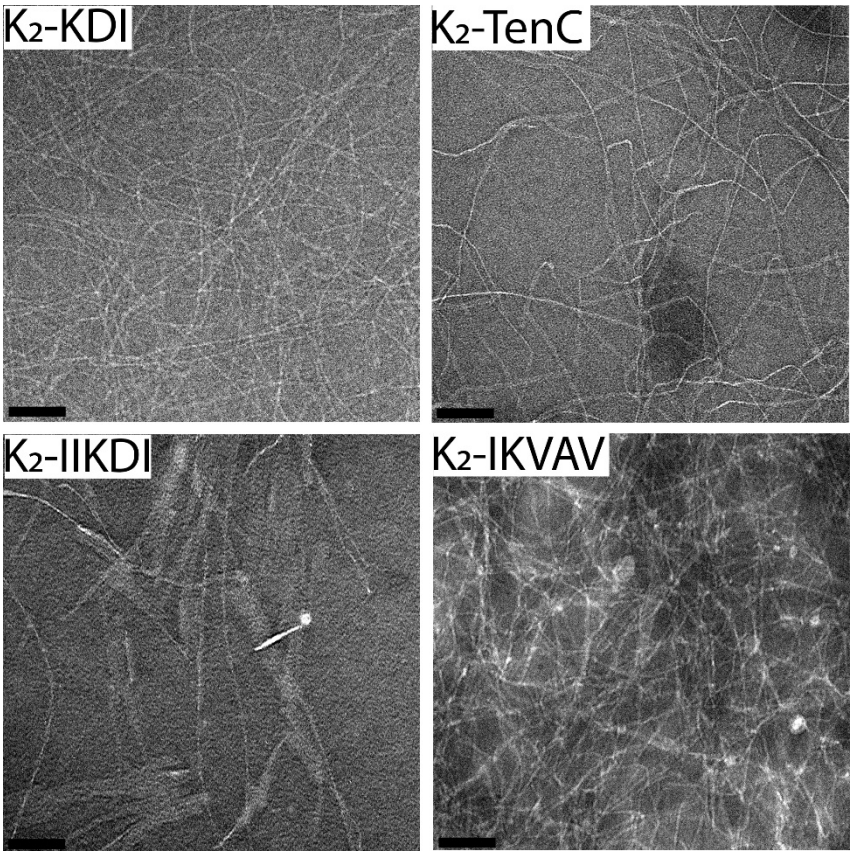

Figure S3. TEM images of functionalized K_2 MDPs. K_2-KDI, K_2-TenC, and K_2-IIKDI at 0.01% peptide in water. K_2-IKVAV at 0.02% peptide in water. Scale bar 100 nm.

Table S2. Primary Antibodies for immunostaining of sciatic nerves

Antibody	Clone	Isotype	Dilution	Brand	Catalog #
Tubulin β-III	TUJ1	Mouse IgG2a, κ	1:1000	Biolegend	801201
GAP-43	Polyclonal	Rabbit	1:500	Abcam	ab16053
CD68	Polyclonal	Rabbit IgG	1:500	Abcam	ab125212

Table S3. Primary Antibodies for immunostaining of sciatic nerves

Host	Isotype	Reactivity	Conjugate	Dilution	Brand	Catalog #
Goat	IgG	Mouse	Alexa Fluor 488	1:500	Invitrogen	A11001
Donkey	IgG	Rabbit	Alexa Fluor 488	1:500	Invitrogen	A21206
Goat	IgG	Mouse	Alexa Fluor 568	1:500	Invitrogen	A11004
Donkey	IgG	Rabbit	Alexa Fluor 568	1:500	Invitrogen	A10042

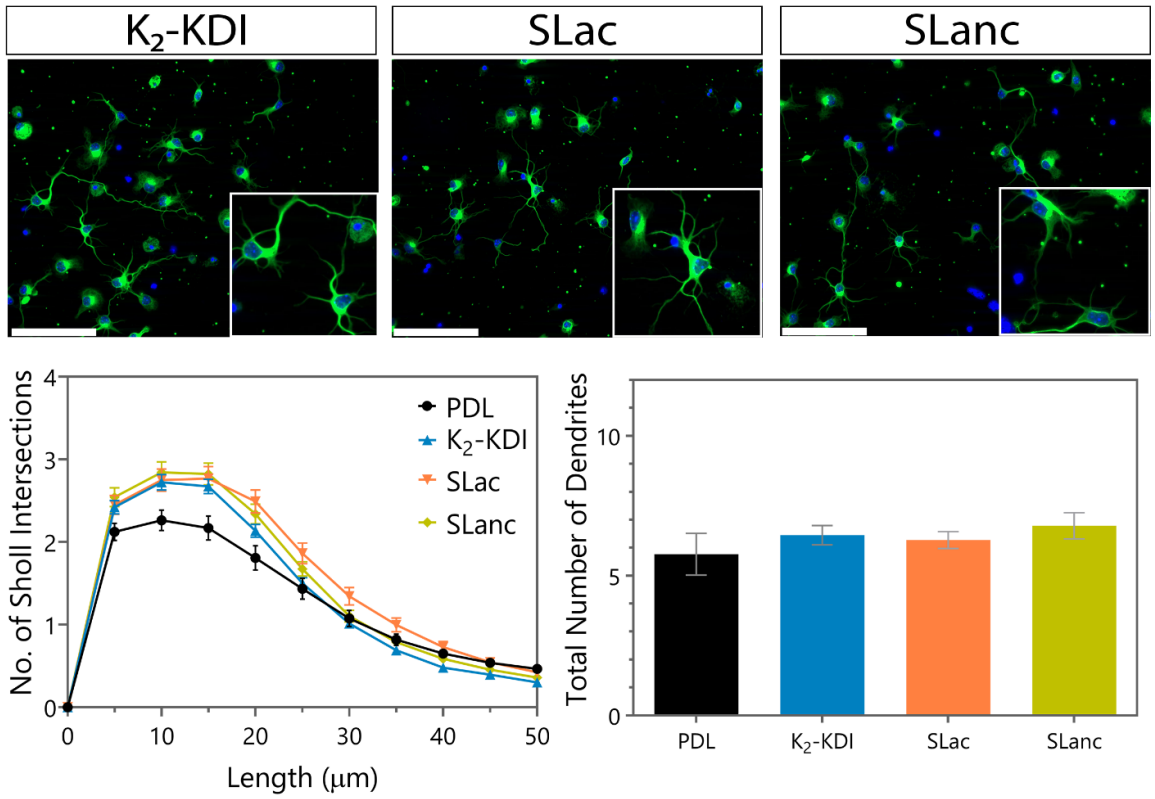

Figure S4. Primary neuronal culture on peptide-coated coverslips for K_2-KDI, SLac and SLanc. Scale bar: 100 µm. Sholl analysis and total number of neurites showed no significant differences in comparison with PDL. Error bars represent SEM. n=24 images from 4 different experiments.

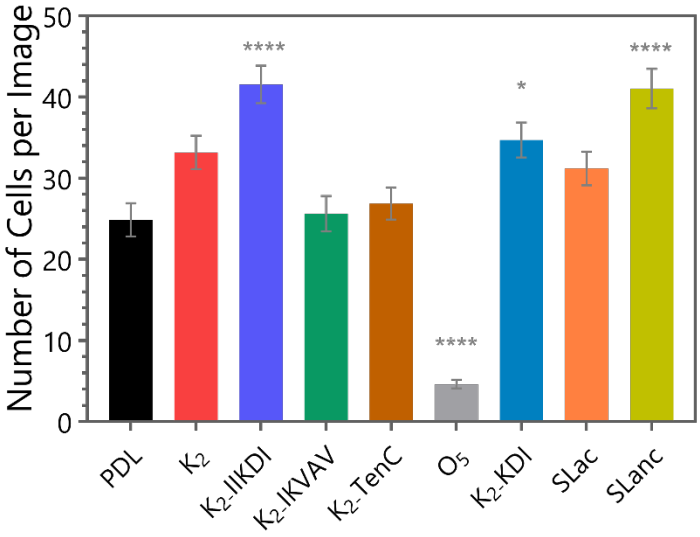

Figure S5. Number of cells per field of view when cultured on peptide-coated glass surfaces. Cell number was higher than the PDL control for surfaces coated with K_2-IIKDI, K_2-KDI, and SLanc and lower for surfaces treated with O_5. * p-value <0.05, **** p-value < 0.0001. Error bars represent SEM. n=24 images from 4 different experiments.

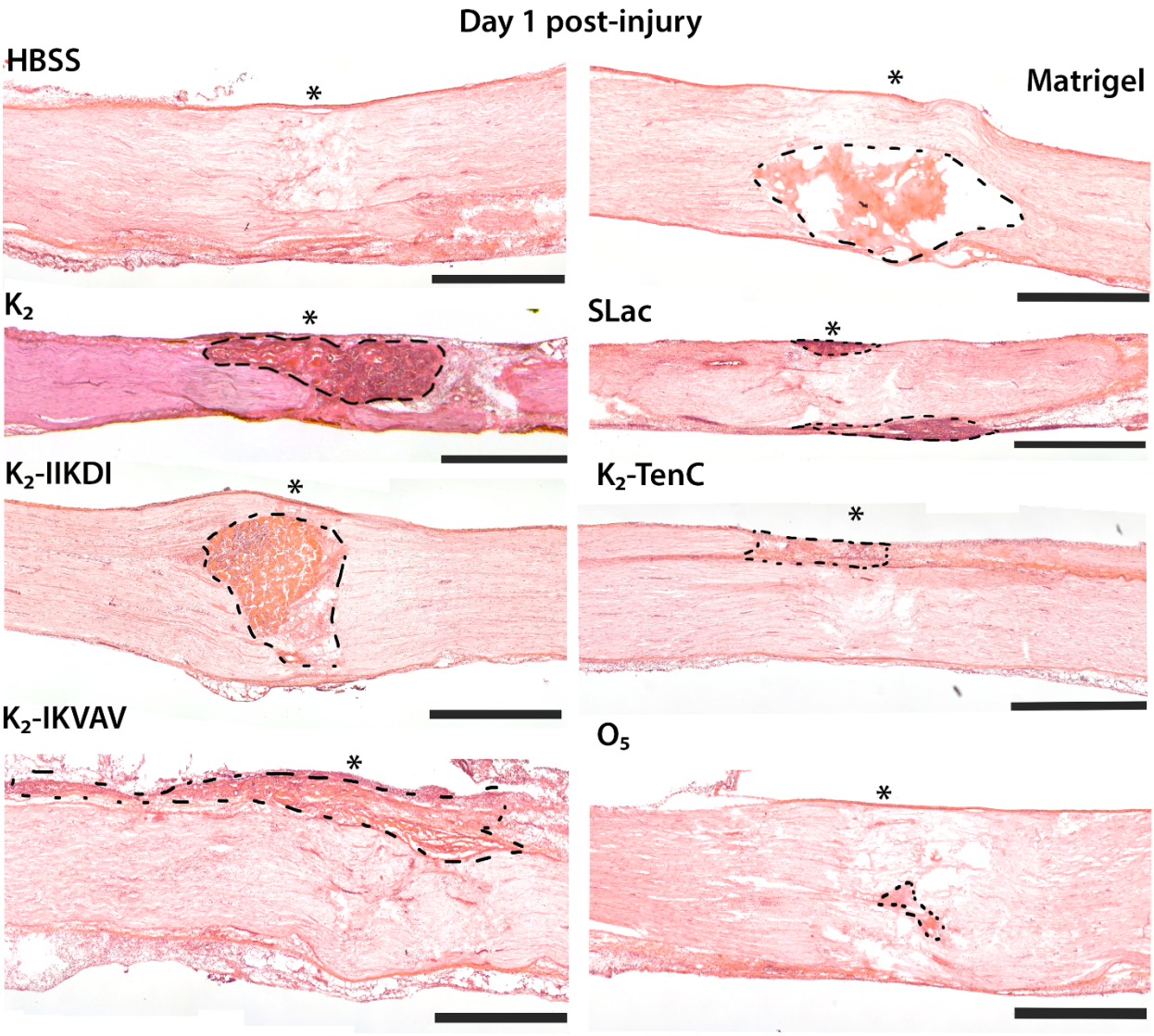

Figure S6. H&E staining of representative sciatic nerves one day post-injury injected with the different treatment groups. Scale bar = 1 mm.

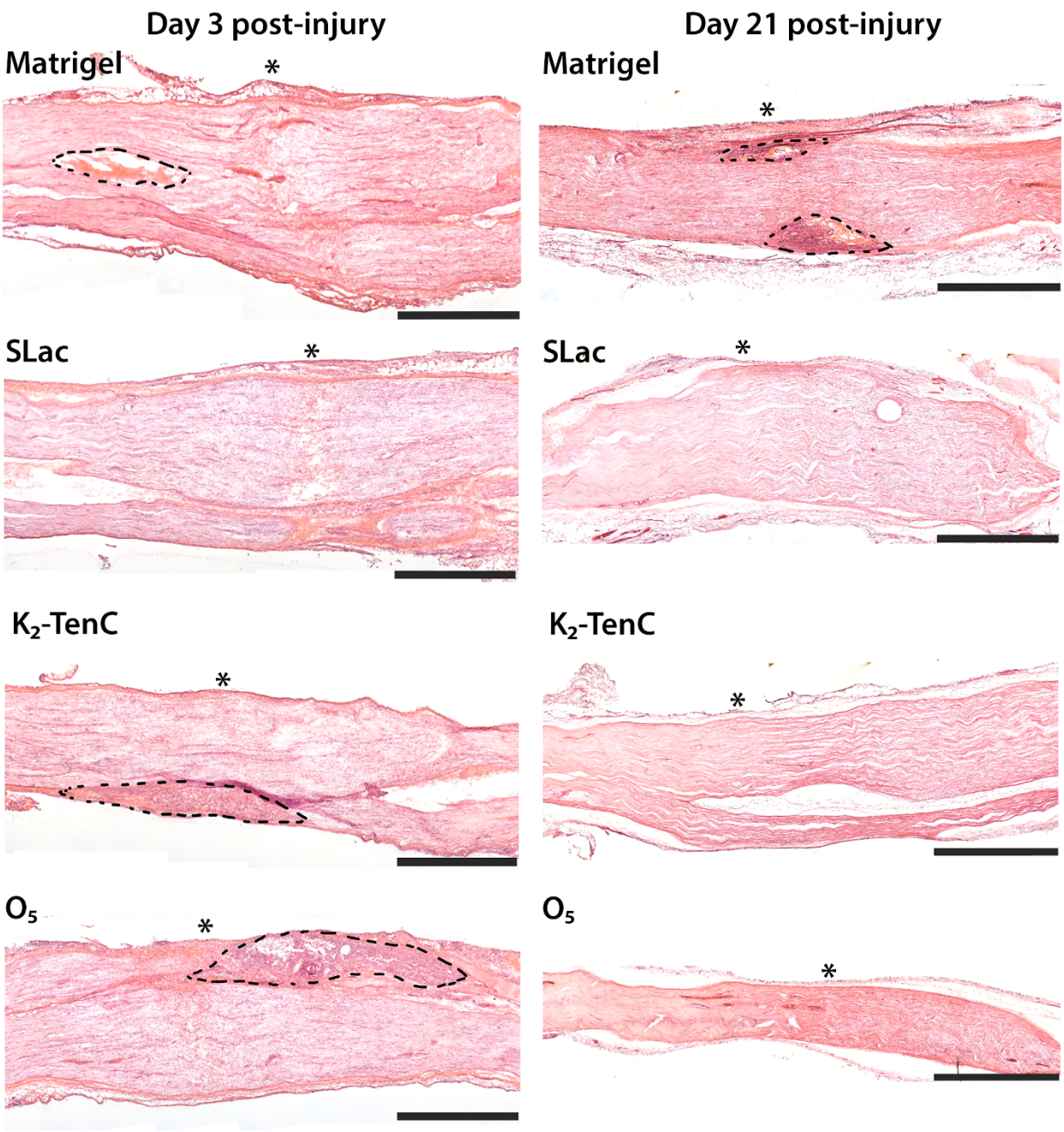

Figure S7. H&E staining of representative sciatic nerves at three- and 21-days post-injury treated with Matrigel, SLac, K_2-TenC and O_5. Scale bar 1 mm.

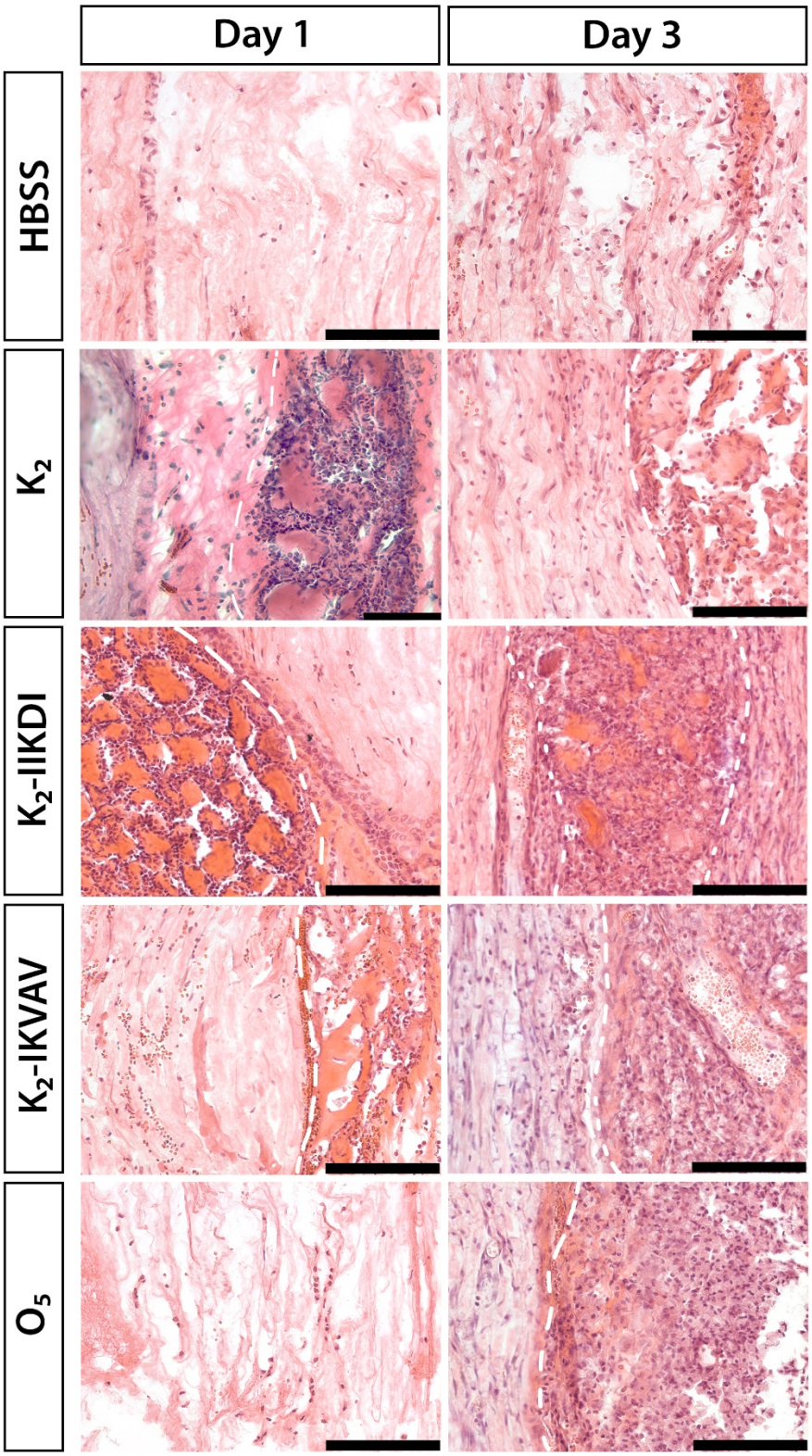

Figure S8. Higher magnification images of H&E stained sciatic nerves one and three days-post injury showing the higher cellular infiltration in the MDP hydrogels. Scale bar: 100 μm.

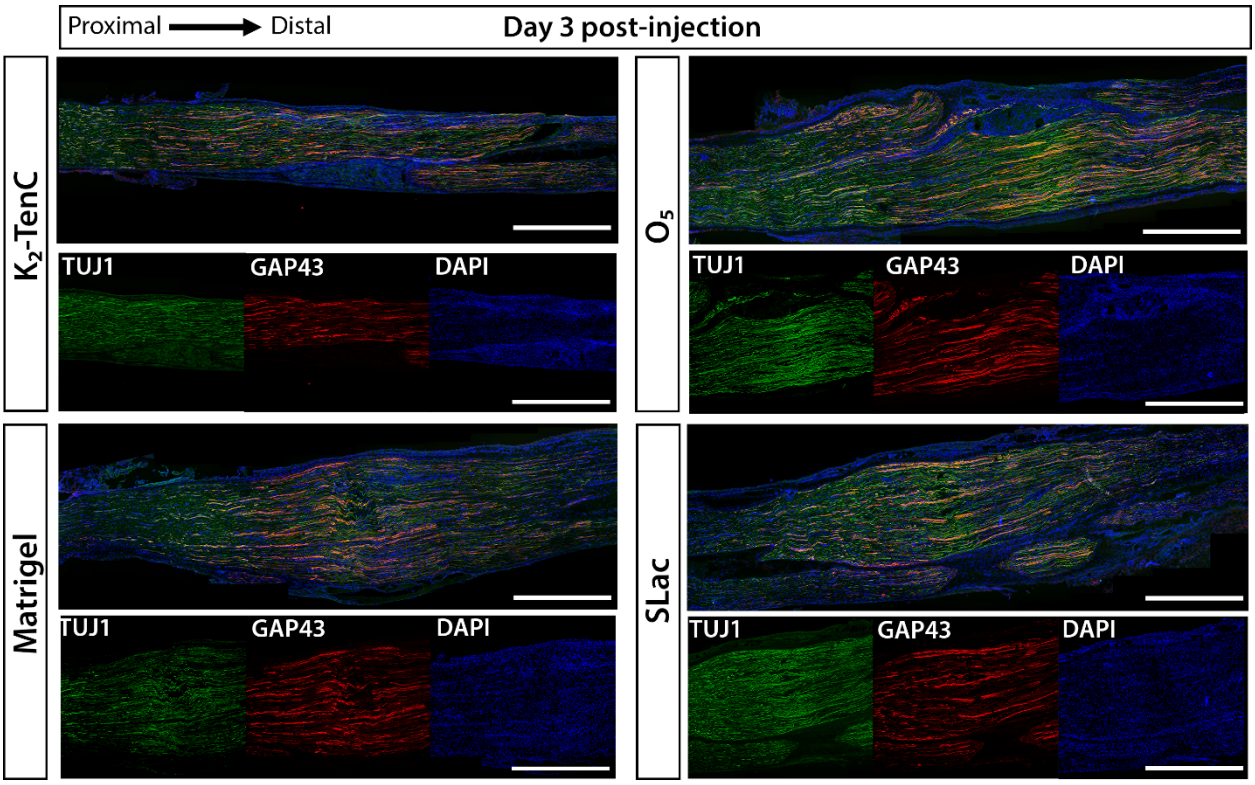

Figure S9. Immunostaining for TUJ1 (axons in green) GAP43 (regenerating axons in red) and DAPI (cell nuclei) in the sciatic nerves treated with the different groups at 3 days post-injury. High levels of GAP43 were observed across all treatments, suggesting no inhibitory effects on acute axonal outgrowth. Scale bar: 1 mm.

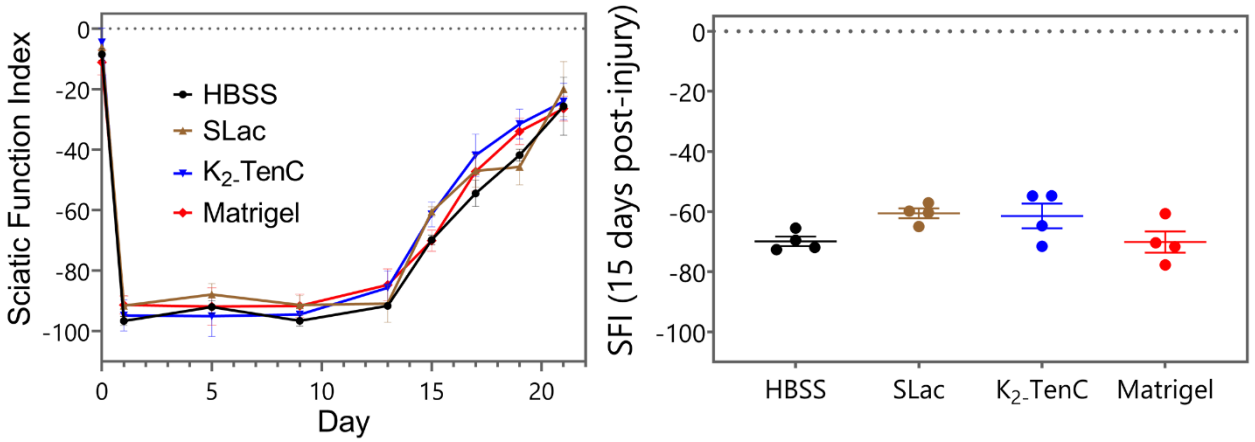

Figure S10. Sciatic Function Index for rats treated with Matrigel, SLac and K_2-TenC. No significant differences in comparison with HBSS are observed. Error bars represent standard deviation. n = 4 mice per group.

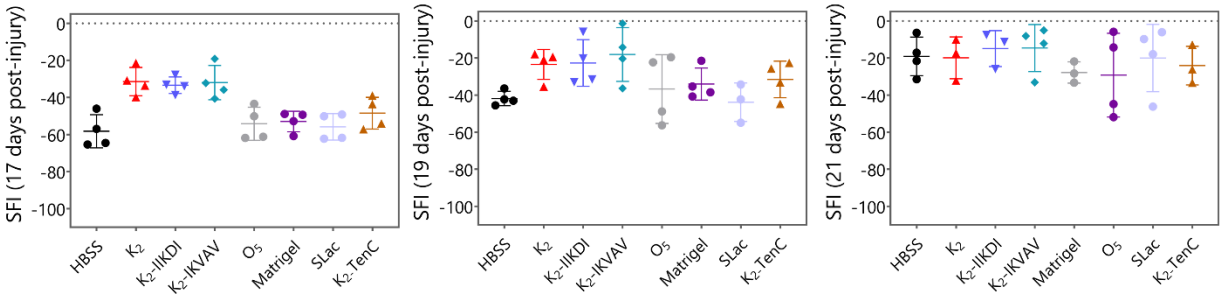

Figure S11. Scatter plots with individual values representation of the SFI 17, 19, and 21 days post-injury. Error bars represent standard deviation. n = 4 mice per group.

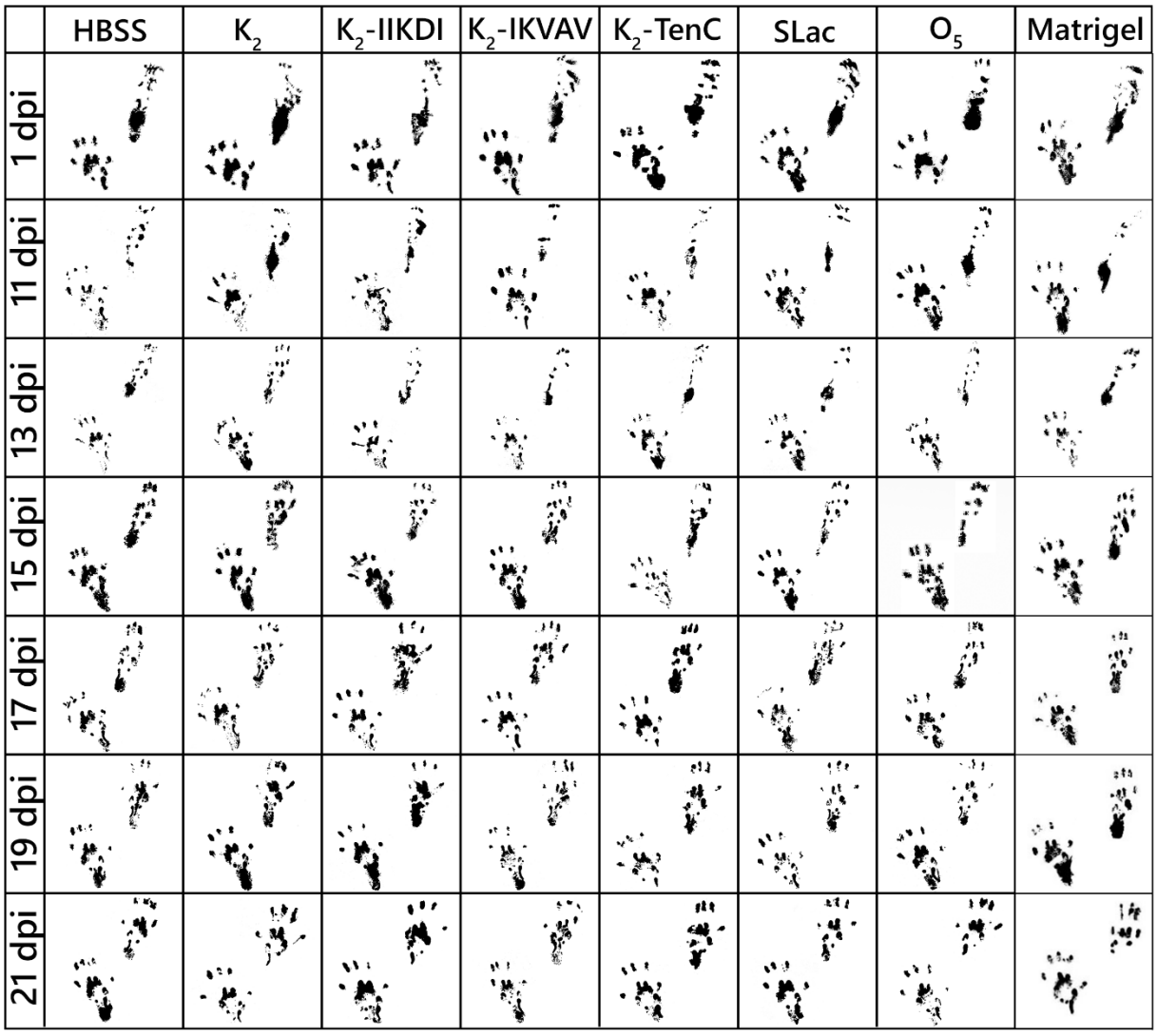

Figure S12. Representative footprints of animals after sciatic nerve crush injury at different days post-injury.

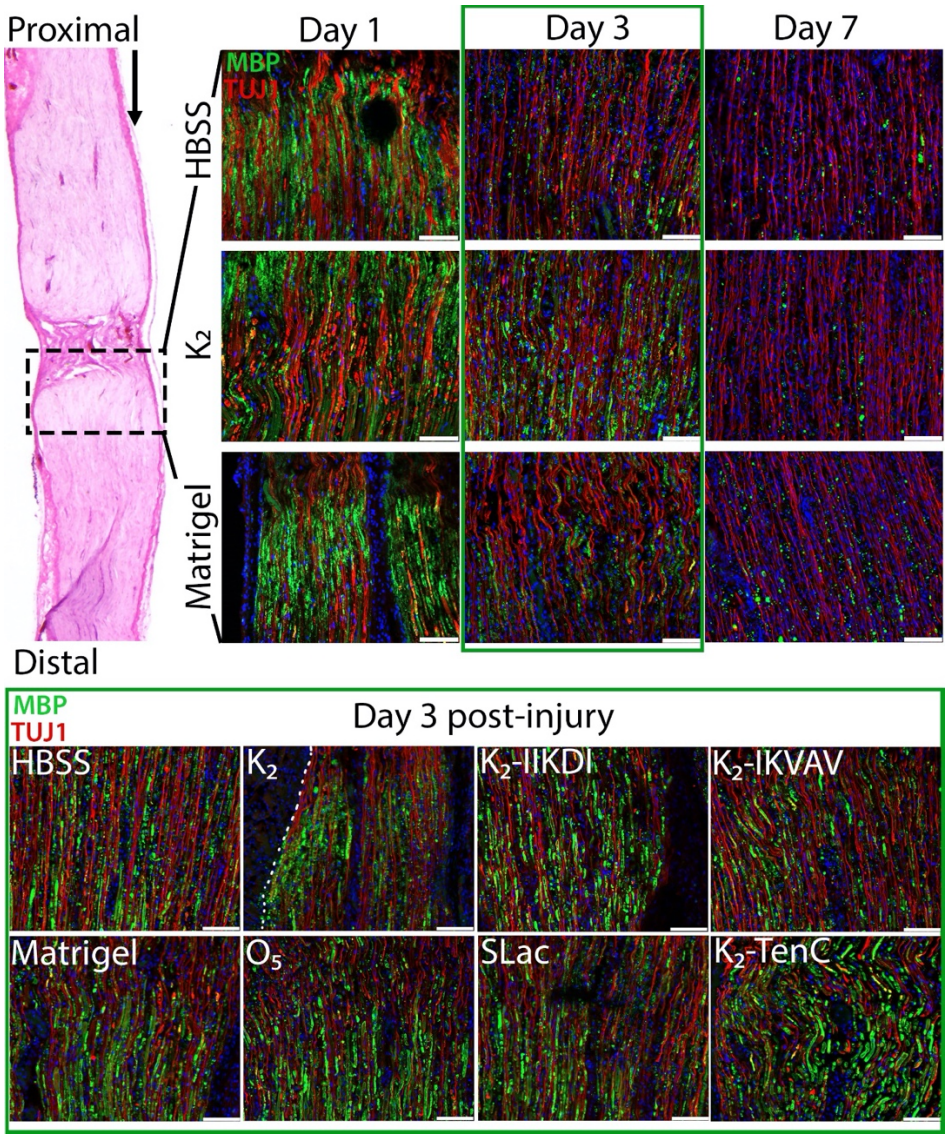

Figure S13. Wallerian degeneration in the distal part of the sciatic nerve after crush injury. Top panel: Myelination in the distal nerve adjacent to the injury site at day 1-, 3-, and 7-post injury. Demyelination is observed at day 3 and progresses by day 7. Bottom panel: Myelination of sciatic nerves at day 3 post injury treated with the HBSS, Matrigel, and the diverse MDPs. Green: MBP, Red: TUJ1, Blue: DAPI. Scale bar 100 μm.

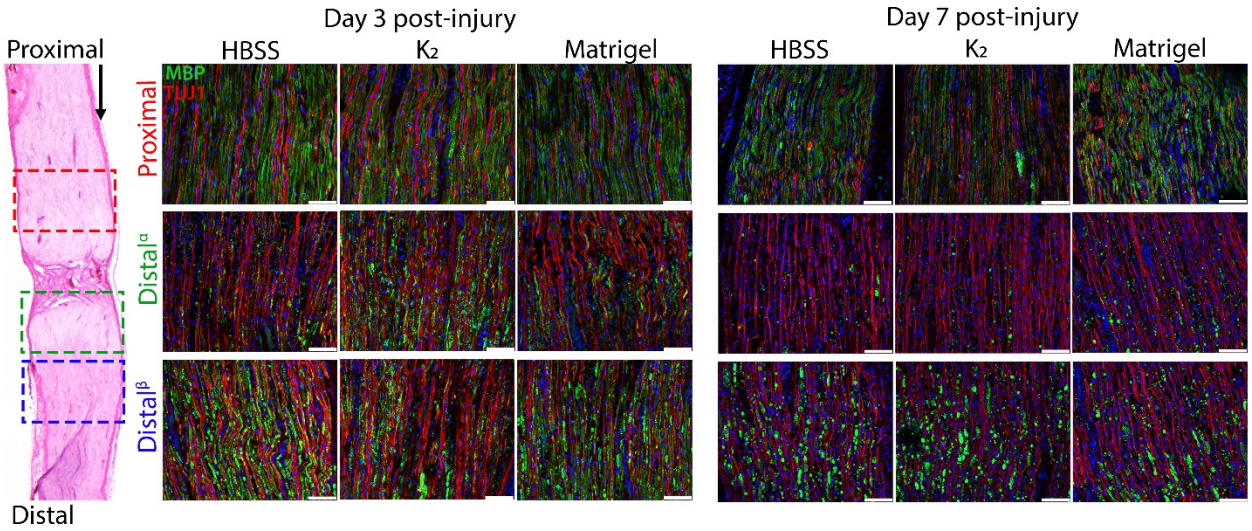

Figure S14. Myelin staining of sciatic nerves in the proximal, $^{\alpha}$ adjacent distal and $^{\beta}$ farther distal three and seven days post-injury. Green: MBP, Red: TUJ1, Blue: DAPI. Scale bar 100 μm.

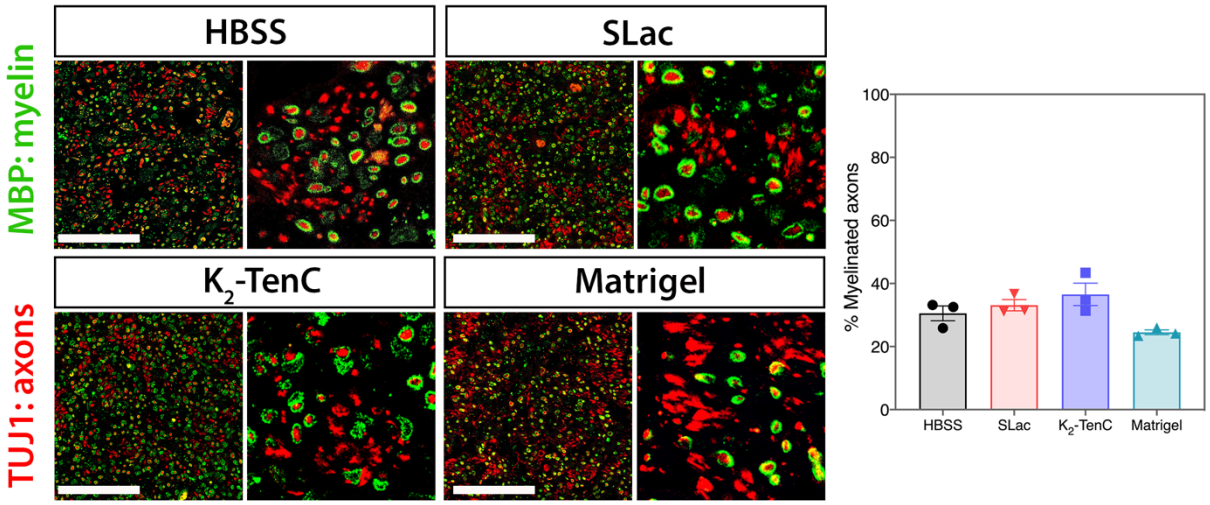

Figure S15. Representative images and quantification of myelinated axons of sciatic nerve cross-sections stained for axons (red) and myelin (green) 15 days post-injury. Scale bar 100 μm. No significant differences in fraction of myelinated axons for nerves treated with SLac, K_2-TenC, or Matrigel compared to control. Error bars represent SEM. n = 3 animals per group.

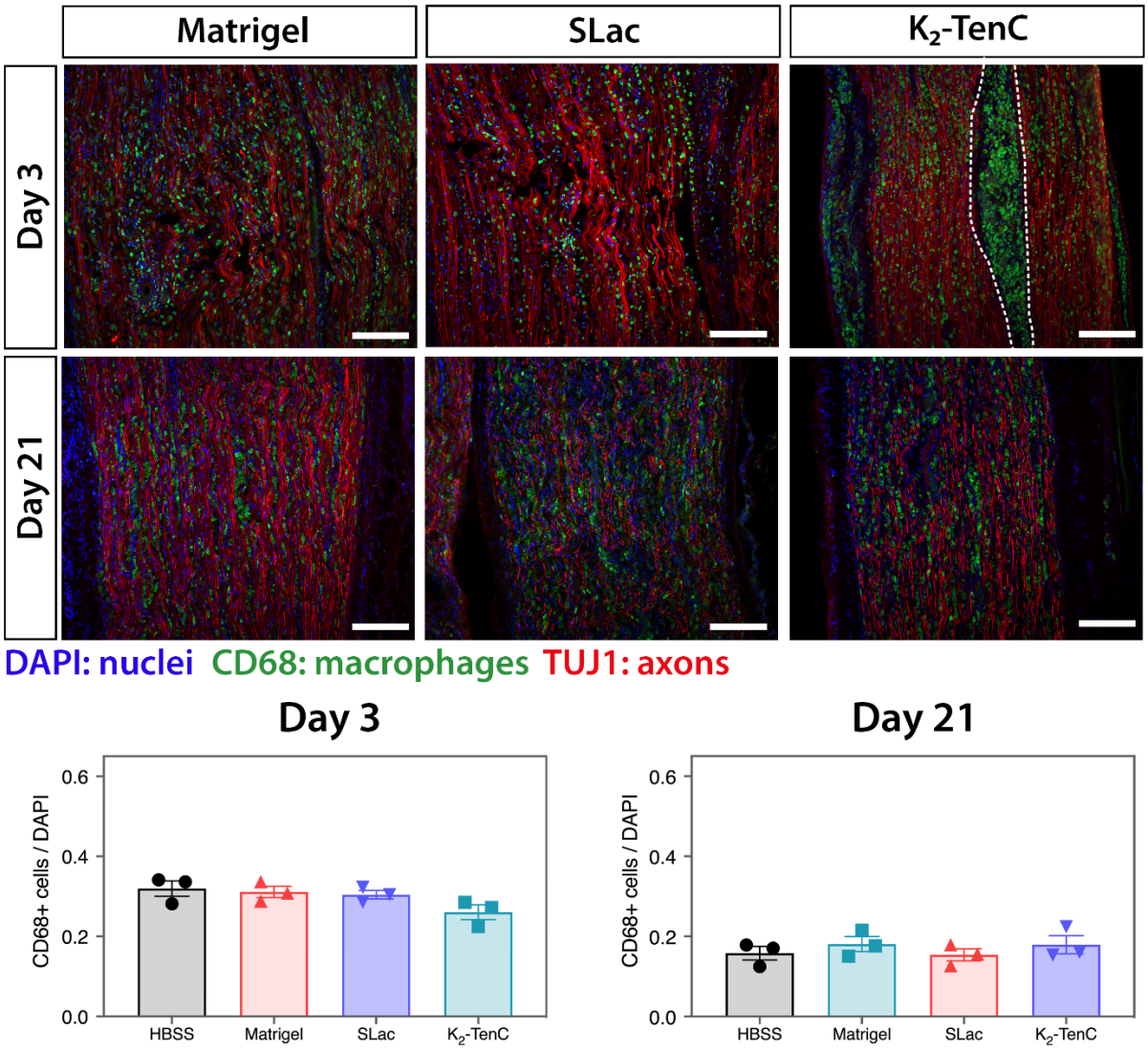

Figure S16. Macrophage infiltration in sciatic nerves after 3 and 21-days post-injury. CD68 (green) for macrophages and TUJ1 (red) for axons. Scale bar 200 μm. No significant differences in macrophage number for nerves treated with SLac, K_2-TenC, or Matrigel compared to control. Error bars represent standard deviation. n = 3 animals per group.

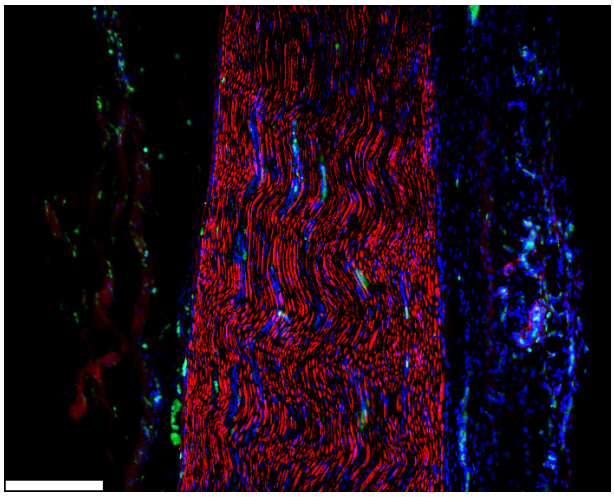

Figure S17. Uninjured proximal nerve stained for TUJ1 (axons) in red, CD68 (macrophages) in green, and DAPI (cell nuclei) in blue. Scale bar 200 μm.