

Supporting Information

Synthetic hookworm-derived peptides are potent modulators of primary human immune cell function that protect against experimental colitis *in vivo*

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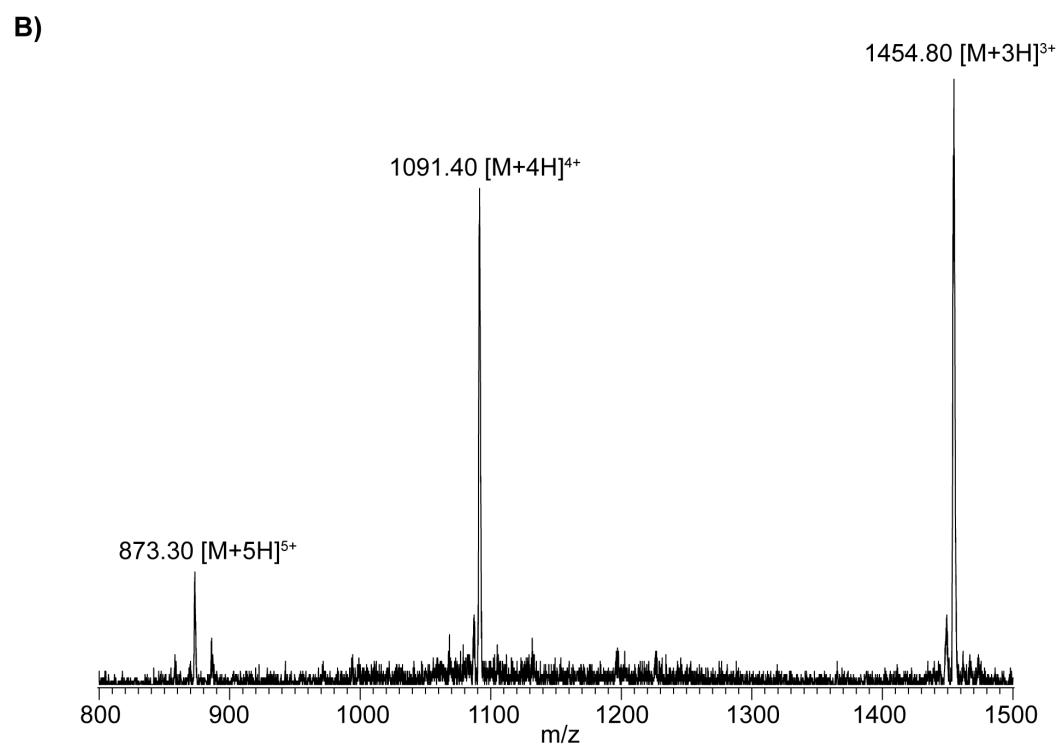
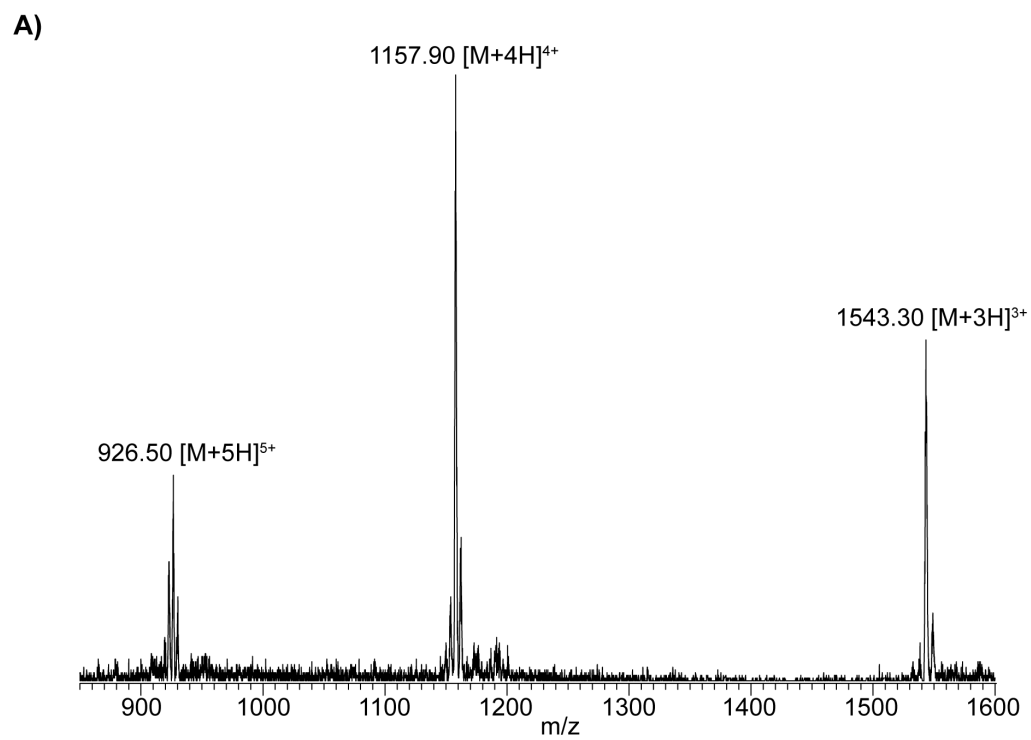


Figure S1: Electrospray ionisation mass spectrometry spectra of final peptides **A)** Acan1 and **B)** Nak1

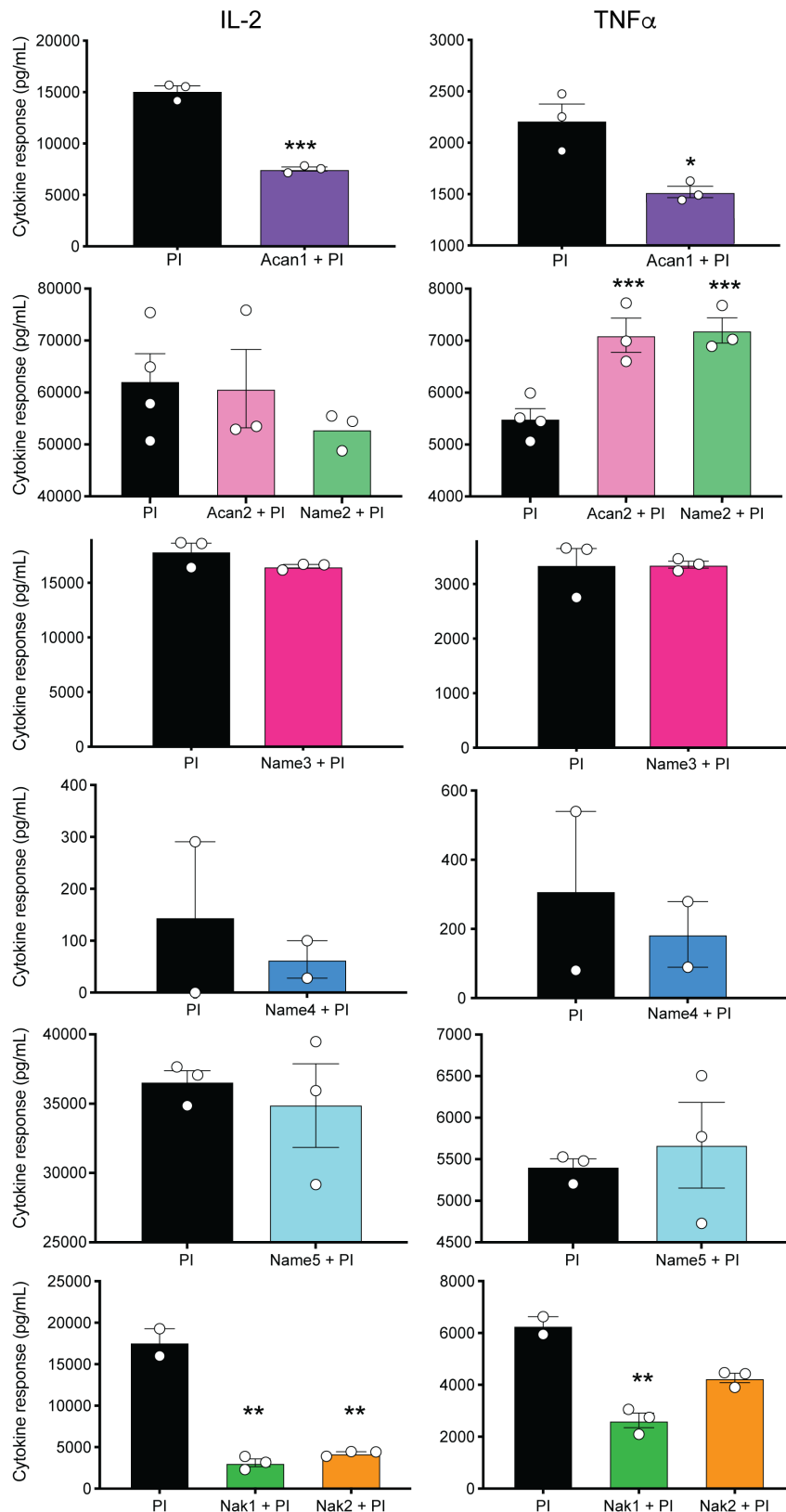
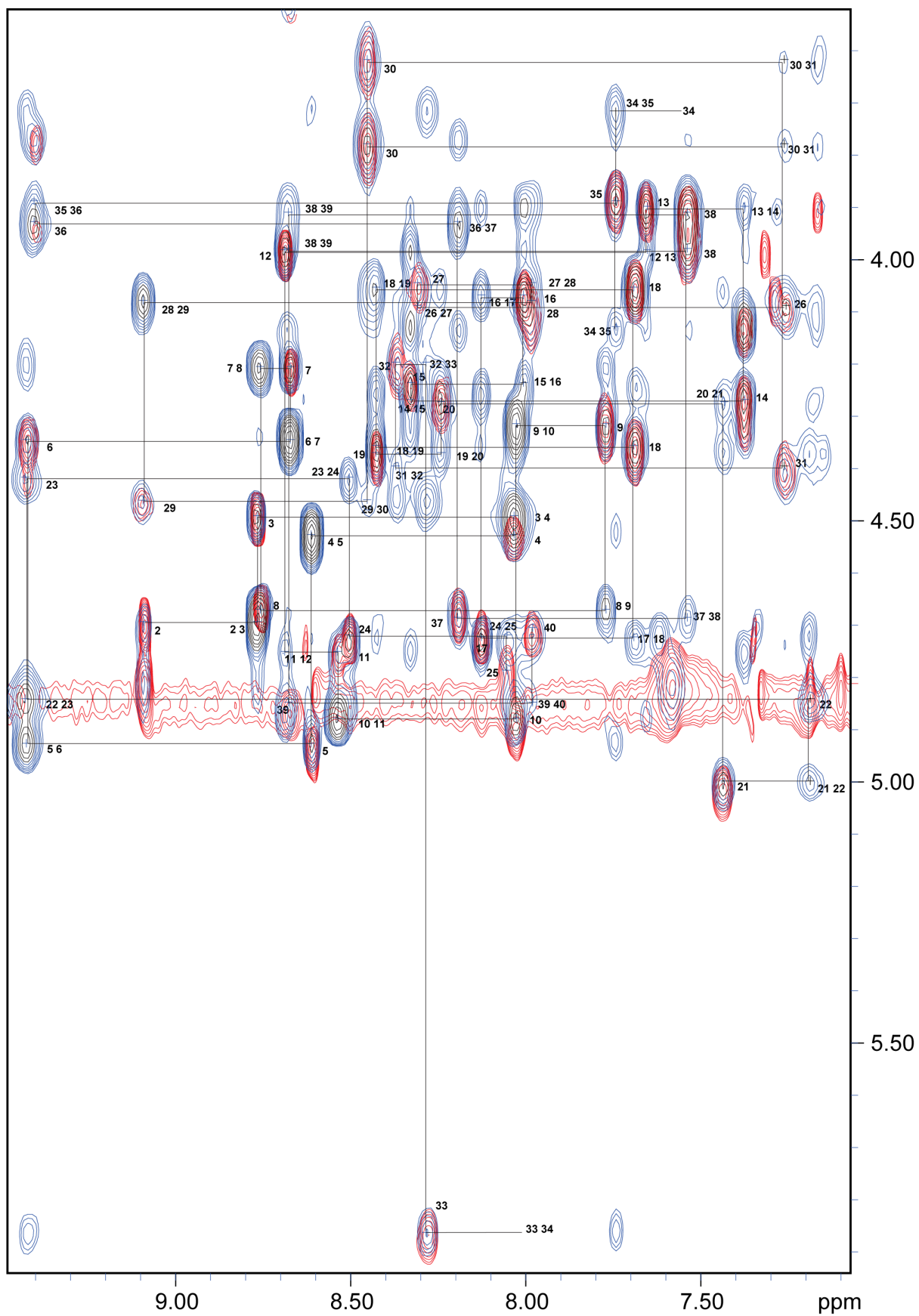


Figure S2: Preliminary functional cytometric bead array (CBA) screening of synthetic hookworm peptides. Human peripheral blood mononuclear cells were stimulated with phorbol 12-myristate 12-acetate (50 ng/mL) + ionomycin (1 μ g/mL) (PI) then treated with 100 μ g/mL hookworm peptides for 24 h. The concentrations of soluble IL-2 and TNF in the culture supernatant were assessed by CBA and flow cytometry (BD LSRFortessa).

A)



B)

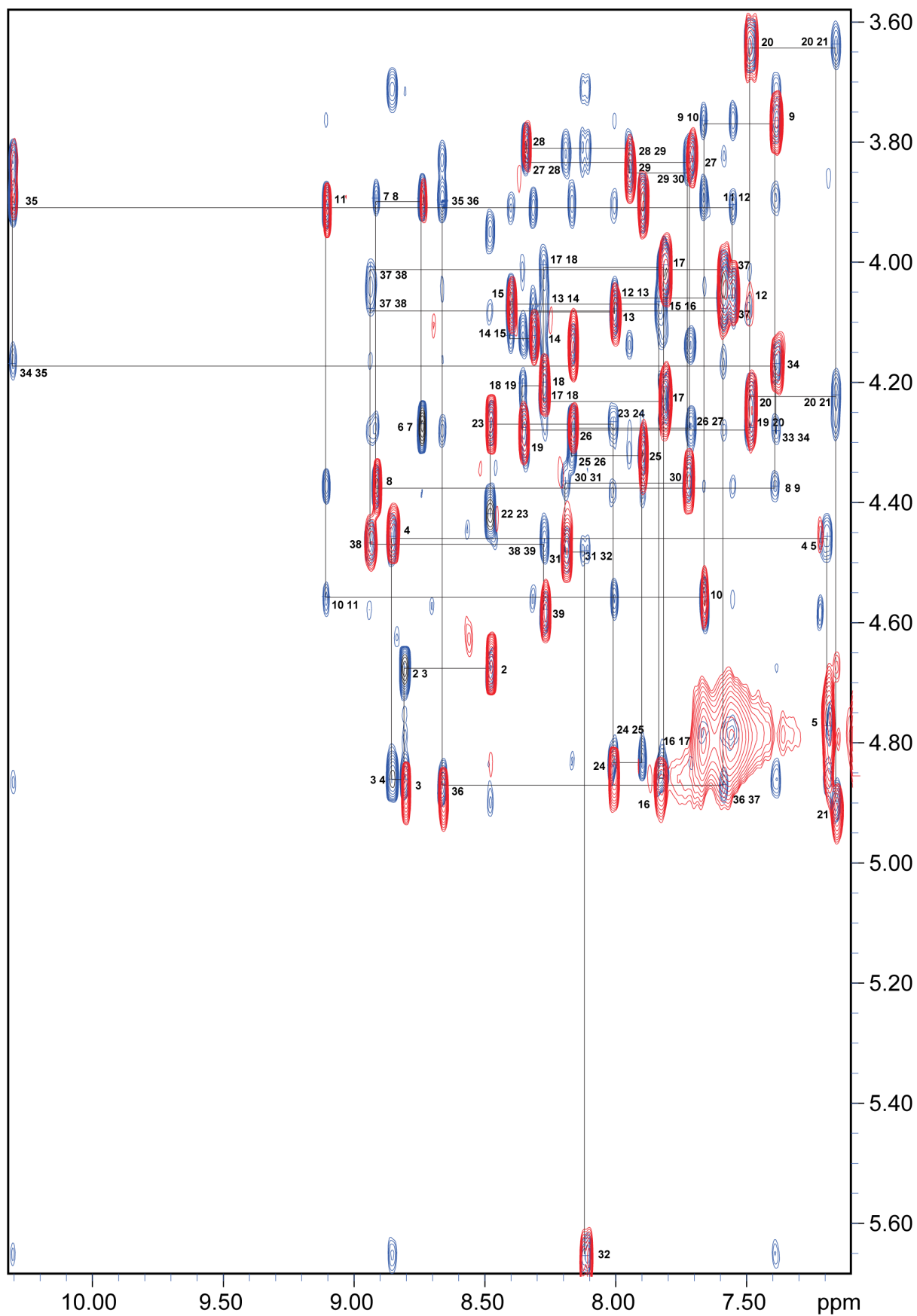


Figure S3: The NOESY and TOCSY spectra overlay of **A)** Acan1 and **B)** Nak1. Hookworm peptide was dissolved in 90% H₂O and 10% D₂O to give a final concentration of 0.5 mM, with data acquired using a Bruker Avance 600 MHz NMR spectrometer. All Spectra were recorded at 298 K. Residue numbers and lines highlight the sequential walk between intra H_α-H_N peaks and sequential H_α-H_{N+1} peaks.

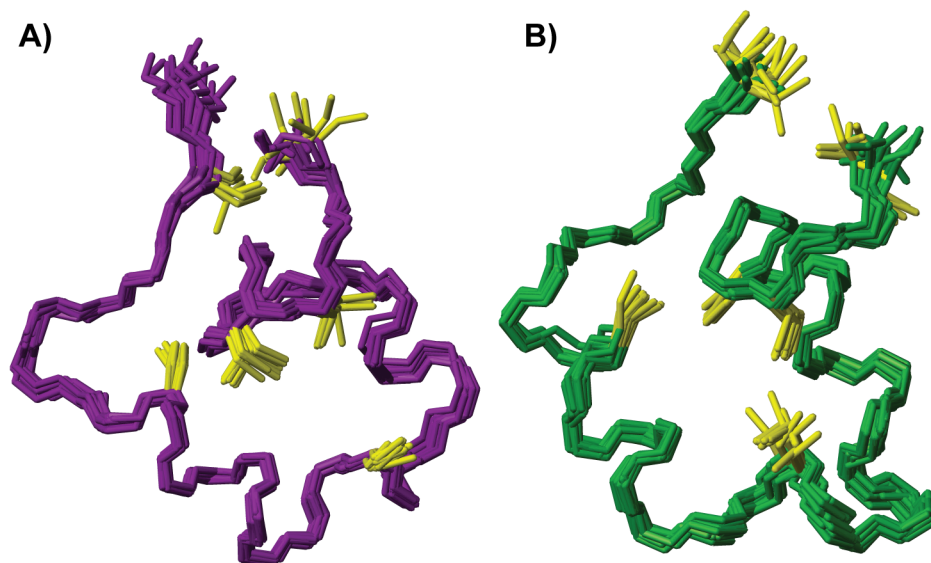


Figure S4: Solution NMR structures of Acan1 and Nak1. The 15 lowest energy structures of **A)** Acan1 and **B)** Nak1 calculated using CYANA without enforcing disulfide bonds. Cysteine side chains are shown in yellow. Images were produced in MOLMOL.

Table S1. NMR distance and dihedral statistics for Acan1 and Nak1

	Acan1	Nak1
Distance restraints		
Intra residual ($i-j = 0$)	167	205
Sequential ($i-j = 1$)	175	193
Medium range ($i-j < 5$)	116	154
Long range ($i-j \geq 5$)	63	74
Hydrogen bonds (HB)	14 (for 7 HB)	36 (for 18 HB)
Total	535	662
Dihedral angle restraints		
Φ	36	29
χ^1	13	19
Ψ	34	29
Total	83	77
Violations from experimental restraints		
NOE violations exceeding 0.2 Å	3	0
Dihedral violations exceeding 2.0°	2	0
Atomic RMSD (Å) ^		
Backbone atoms	0.89 ± 0.24	0.73 ± 0.17
Heavy atoms	1.62 ± 0.20	1.43 ± 0.17
Molprobit		
Clash Score, all atoms	15.24 ± 3.60	14.09 ± 3.5
Poor rotamers	0.300 ± 0.47	0.4 ± 0.5
Ramachandran favoured (%)	95.29 ± 2.73	90.13 ± 14.19
Ramachandran outliers (%)	98.81 ± 1.59	99.59 ± 0.98
Molprobit score	1.83 ± 0.14	1.80 ± 0.19
Molprobit score percentile	82.95 ± 6.32	84.35 ± 7.5
Bad backbone bonds/angles	0 / 0.05 ± 0.22	0 / 0

^ Atomic RMSD calculated over residues Cys3 - Cys40 for Acan1 and Asp3 – Gly37 for Nak1

Table S2. Structural statistics for Acan1 and Nak1 from CYANA

Acan1	DB 1	DB 2	DB 3	Violations	Target function avg	Avg backbone RMSD Å
No bonds	-	-	-	0	0.12	0.54 +/- 0.31
1	3-40	11-33	20-37	0	7.99E-02	0.21 +/- 0.05
2	3-40	11-37	20-33	4 (2 x Cys)	0.53	0.32 +/- 0.07
3	3-40	11-20	37-33	3 (2 x Cys)	0.28	0.53 +/- 0.11
4	3-33	11-40	20-37	3 (1 x Cys)	0.71	0.30 +/- 0.11
5	3-33	11-37	20-40	3	0.30	0.26 +/- 0.05
6	3-33	11-20	37-40	1	0.27	0.86 +/- 0.27
7	3-37	11-33	40-20	3 (1 x Cys)	0.50	0.30 +/- 0.05
8	3-37	11-20	33-40	4 (1 x Cys)	0.59	0.70 +/- 0.22
9	3-37	11-40	33-20	4 (2 x Cys)	0.92	2.06 +/- 0.25
10	3-11	40-33	20-37	3 (3 x Cys)	1.42	0.81 +/- 0.16
11	3-11	40-37	20-33	11 (4 x Cys)	1.59	1.24 +/- 0.47
12	3-11	40-20	37-33	4 (1 x Cys)	0.68	0.61 +/- 0.19
13	3-20	11-33	40-37	3 (1 x Cys)	0.55	0.53 +/- 0.22
14	3-20	11-37	40-33	5 (1 x Cys)	0.69	0.60 +/- 0.09
15	3-20	11-40	33-37	8 (3 x Cys)	1.44	0.54 +/- 0.22

Nak1	DB 1	DB 2	DB 3	Violations	Target function avg	Avg backbone RMSD Å
No bonds	-	-	-	2 (1 x Cys)	0.56	0.40 +/- 0.08
1	1-39	10-32	19-36	3 (1 x Cys)	0.46	0.43 +/- 0.15
2	1-39	10-36	19-32	7 (3 x Cys)	0.96	0.37 +/- 0.09
3	1-39	10-19	36-32	6 (4 x Cys)	1.31	0.32 +/- 0.06
4	1-32	10-39	19-36	9 (2 x Cys)	1.32	0.26 +/- 0.09
5	1-32	10-36	19-39	7 (4 x Cys)	0.69	0.37 +/- 0.09
6	1-32	10-19	36-39	12 (7 x Cys)	1.45	0.45 +/- 0.14
7	1-36	10-32	39-19	13 (4 x Cys)	1.68	0.31 +/- 0.09
8	1-36	10-19	32-39	15 (6 x Cys)	1.49	0.40 +/- 0.09
9	1-36	10-39	32-19	15 (7 x Cys)	1.62	0.47 +/- 0.09
10	1-10	39-32	19-36	6 (2 x Cys)	1.12	0.22 +/- 0.08
11	1-10	39-36	19-32	4 (2 x Cys)	0.69	0.28 +/- 0.21
12	1-10	39-19	36-32	8 (5 x Cys)	1.25	0.39 +/- 0.12
13	1-19	10-32	39-36	17 (8 x Cys)	2.08	0.33 +/- 0.12
14	1-19	10-36	39-32	20 (9 x Cys)	2.68	0.46 +/- 0.10
15	1-19	10-39	32-36	22 (13 x Cys)	3.25	0.28 +/- 0.10

DB – Disulfide bond

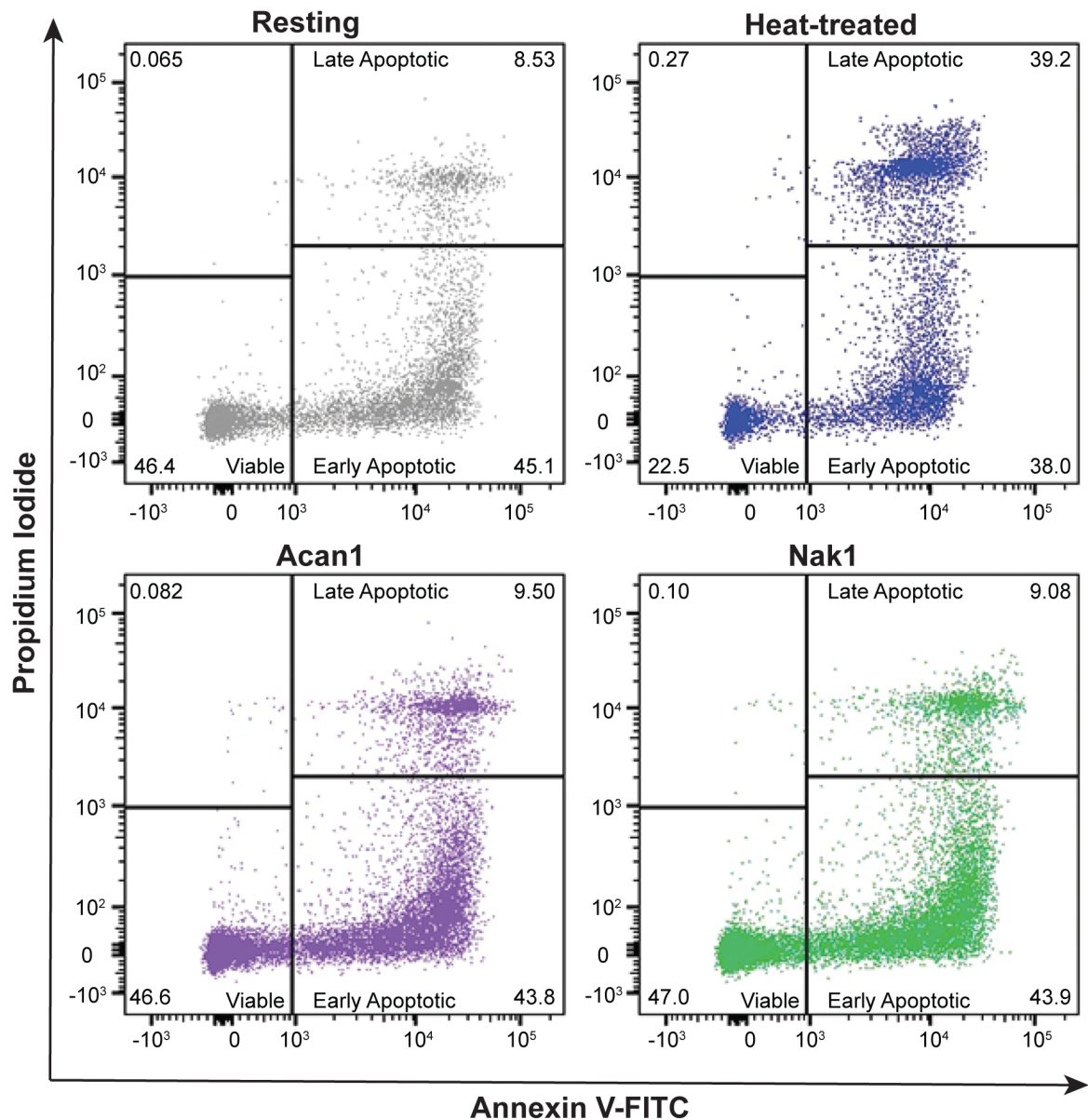


Figure S5: Annexin V apoptosis assay of peripheral blood mononuclear cells (PBMC) incubated with hookworm peptide. Human PBMCs were treated with Acan1 (purple) or Nak1 (green) at 10 $\mu\text{g}/\text{mL}$ for 4 hours at 37°C/10% CO₂. A positive control (blue) was generated by heating cells to 56°C for 20 mins. Following treatment, PBMCs were labelled with Annexin V-FITC and propidium iodide and the percentage of apoptotic and necrotic populations assessed by flow cytometry (BD LSRFortessa).

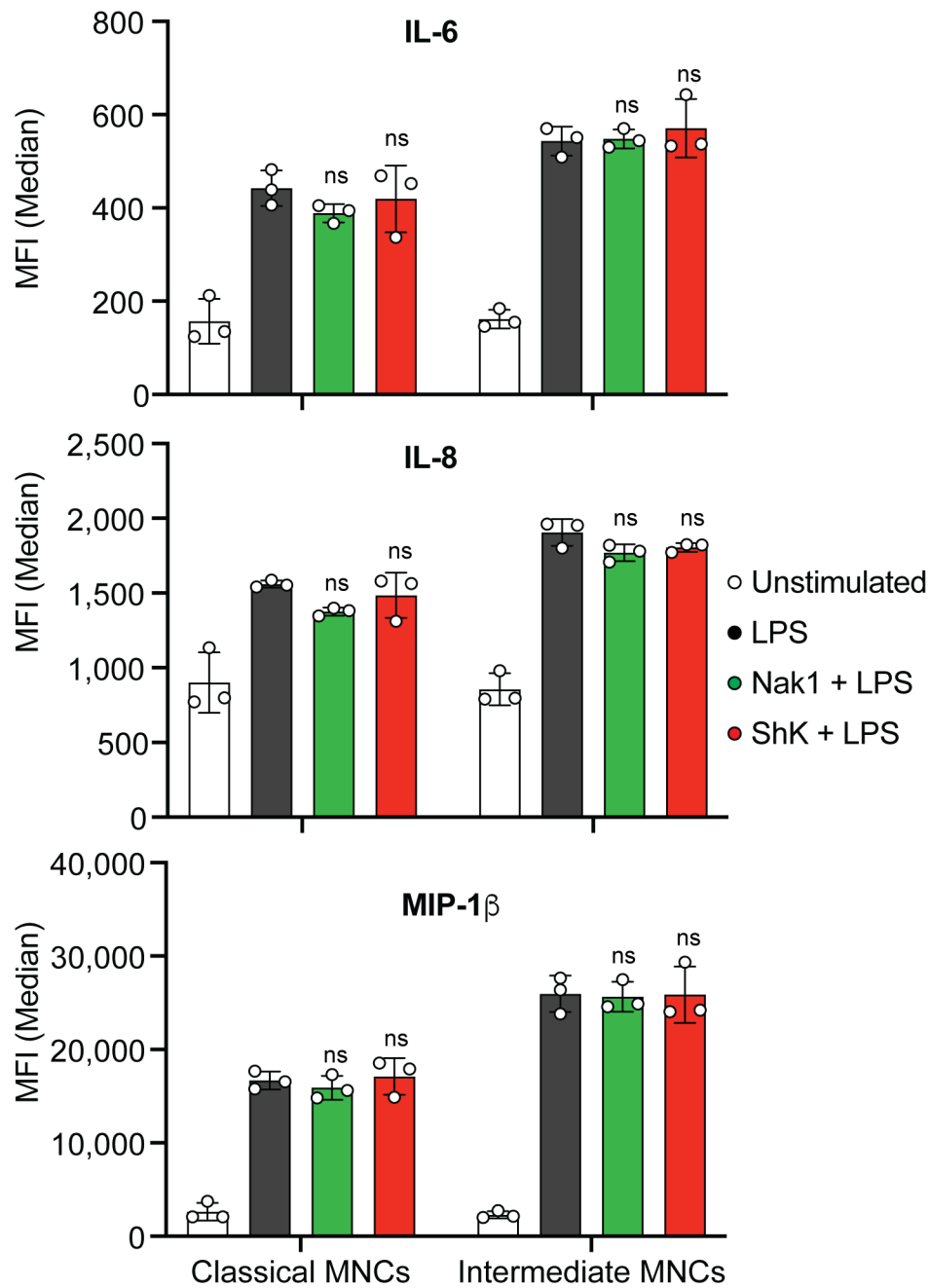


Figure S6: Intracellular cytokine staining for IL-6, IL-8 and MIP-1 β in classical (CD14⁺) or intermediate (CD14⁺/CD16⁺) monocytes (MNCs) exposed to Nak1 or ShK. Human peripheral blood mononuclear cells were stimulated with 10 ng/mL lipopolysaccharide (LPS) then treated with Nak1 (100 μ g/mL) or ShK (10 μ g/mL) peptides for 24 h. Cytokine production was analysed by flow cytometry (BD LSRFortessa). Bars show mean MFI \pm SD. Symbols show replicate values. n = 3; ns, no statistical significance; student's t-test comparing LPS to peptide treatment.

Mixed Lymphocyte Reaction

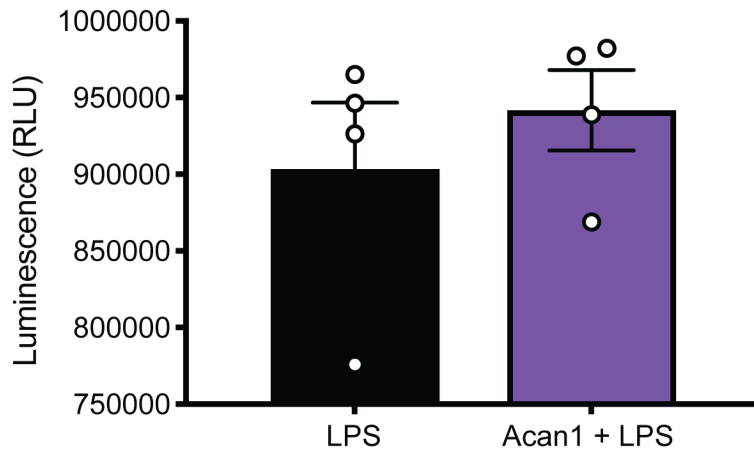


Figure S7: Mixed lymphocyte reaction using Acan1. Monocyte-derived dendritic cells (MoDCs) were generated from peripheral blood CD14⁺ monocytes cultured with granulocyte-macrophage colony stimulating factor and IL-4 for 5 days. On day 5, CD14⁻ MoDCs (1×10^4 cells/well) were stimulated with 100 ng/mL lipopolysaccharide (LPS) then treated with Acan1 (100 μ g/mL) for 24 h. Following incubation, isolated CD3⁺ T cells were added to the samples at a ratio of 10:1 (1×10^5 T cells/well). Mixed cultures were incubated for a further 5 days. Metabolically active cells were then determined as a quantitation of ATP luminescence (RLU) using CellTiter-Glo Luminescent Cell Viability Assay. Bars show mean RLU \pm SD. Symbols show replicate values.

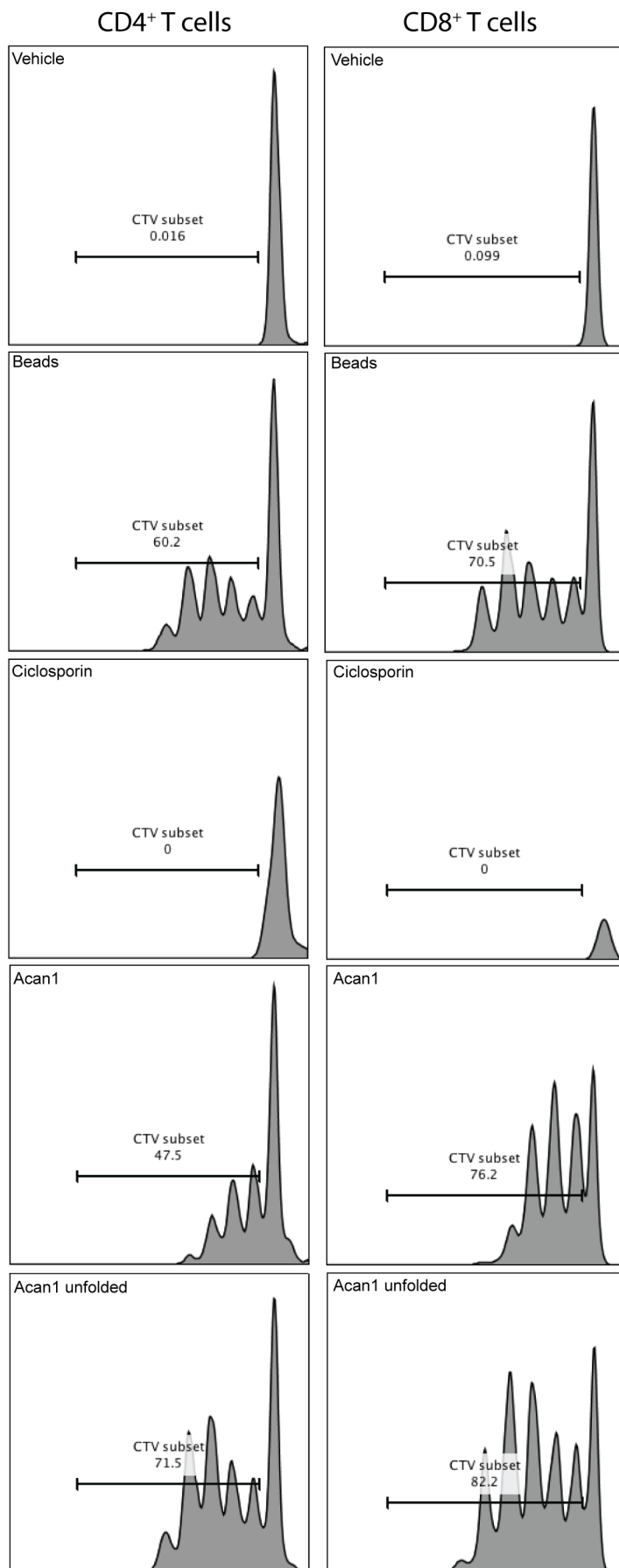


Figure S8: Example gating from CellTrace Violet proliferation assay showing T cell divisions. Purified human T cells were labelled with CellTrace Violet reagent then activated with anti-CD3/anti-CD28 stimulation beads. Activated T cells were treated with 10 µg/mL ciclosporin (suppressive control), 100 µg/mL Acan1, or 100 µg/mL unfolded Acan1 for 4 days at 37°C/5% CO₂. The effect of folded and unfolded peptide on the percentage of live proliferating T cells was assessed by flow cytometry using a BD LSRFortessa (BD Bioscience).

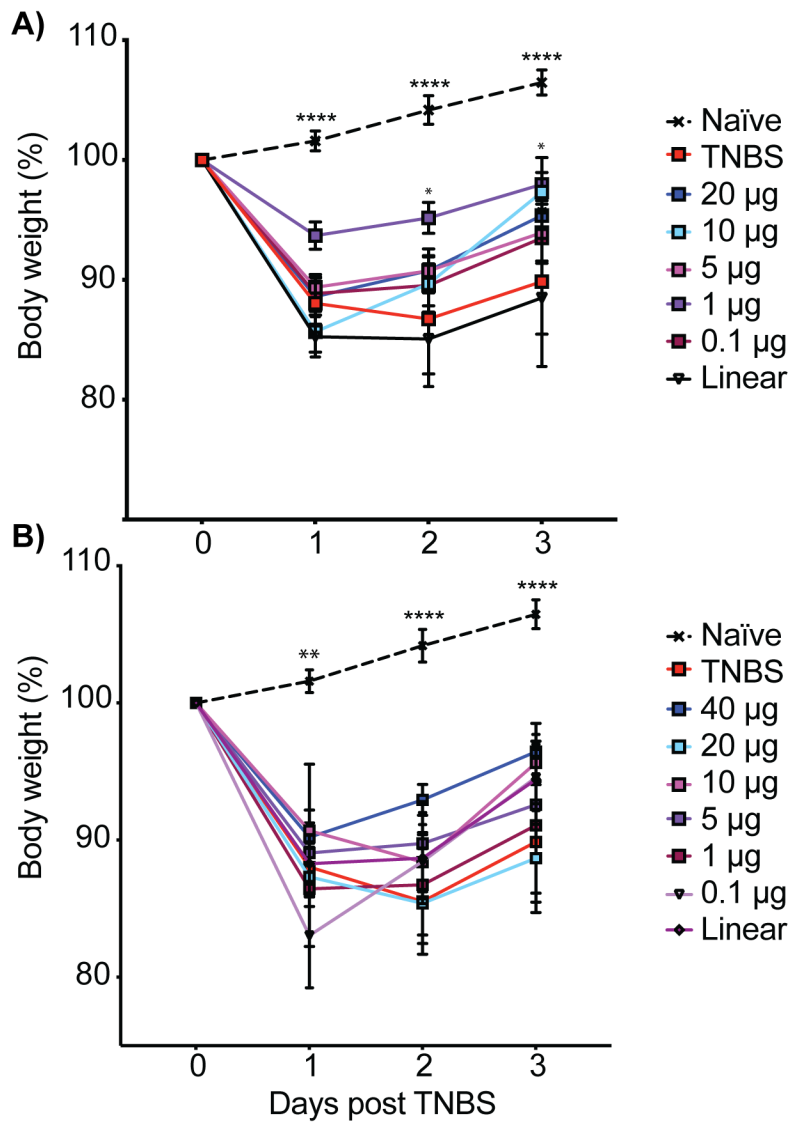


Figure S9: Mouse body weight was recorded daily for the indicated groups. Mice were administered ranging concentrations of **A)** Acan1 or **B)** Nak1 prior to TNBS at day 0 and weighed daily before being euthanised at day four. Data show mean \pm s.e.m. of a representative experiment out of three with $n = 5$. Two-way ANOVA with Dunnett's multiple comparisons test was used to compare each group to vehicle over time; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

