Supplementary Materials for

PEGylated Polyester Nanoparticles Trigger Adverse Events in a Large Animal Model of Trauma and in Naïve Animals: Understanding Cytokine and Cellular Correlations with These Events

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The characterization data for the nanocapsules is summarized in table 1 and table 2 for the hemostatic and control nanoparticles, respectively.

Supplementary Table 1: Summary of hydrodynamic diameter, zeta-potential and peptide density for the hemostatic nanoparticles

Group	Z-Average (nm)	Z-Potential (mV)	Peptide content %
Treatment 1	417.3 ± 12	-14.3 ± 1.02	8.7
Treatment 2	383.1 ± 14	-14.4 ± 0.6	7.6
Treatment 3	414.9 ± 24	-14.3 ± 0.81	7.7
Treatment 4	389.7 ± 6	-13.7 ± 0.95	8.7
Treatment 5	410.8 ± 13	-14.9 ± 0.83	7.0
Treatment 6	567 ± 878	-14.2 ± 1.02	8.4
Treatment 7	432.2 ± 16	-13.4 ± 0.62	5.6
Treatment 8	419.8 ± 10	-13 ± 0.84	6.5
Treatment 9	454.2 ± 28	-13.4 ± 1.03	5.0
Treatment 10	417.2 ± 13	-13.4 ± 0.6	3.4
Treatment 11	468.2 ± 12	-13.1 ± 0.5	5.4
Treatment 12	518.4 ± 64	-11.4 ± 0.84	6.9

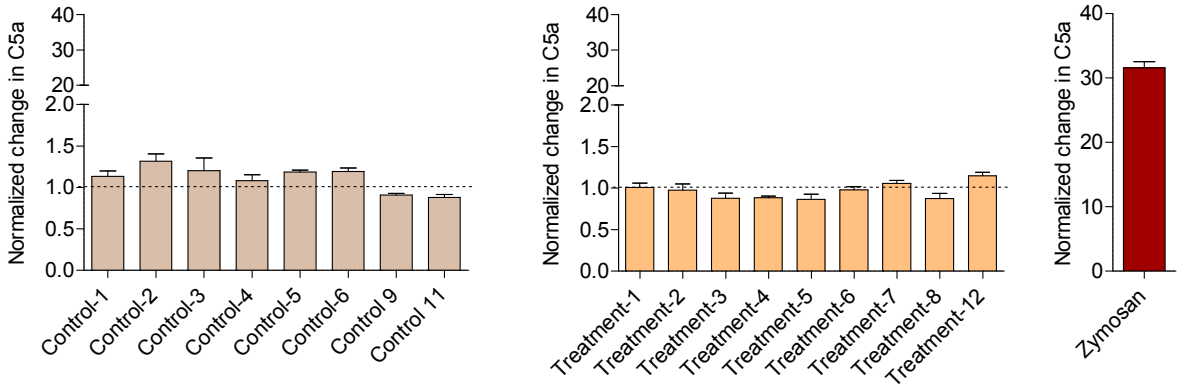
Supplementary Table 2: Summary of hydrodynamic diameter, and zeta-potential for the hemostatic nanoparticles

Group	Z-Average (nm)	Z-Potential (mV)
Control 1	441 ± 32.8	-15.8 ± 0.98
Control 2	449.6 ± 38	-15 ± 1.26
Control 3	397.1 ± 20	-15.6 ± 1.1
Control 4	370.9 ± 4	-15.4 ± 1.17
Control 5	394 ± 35	-14.9 ± 0.61
Control 6	321.6 ± 3	-16 ± 1.02
Control 7	336 ± 2	-15.7 ± 0.79
Control 8	400.6 ± 44	-15.6 ± 0.89
Control 9	353.6 ± 14	-16 ± 1.07
Control 10	362.8 ± 10	-15.1 ± 1.14
Control 11	343.4 ± 15	-16.1 ± 0.76
Control 12	359.6 ± 24	-15.6 ± 0.8

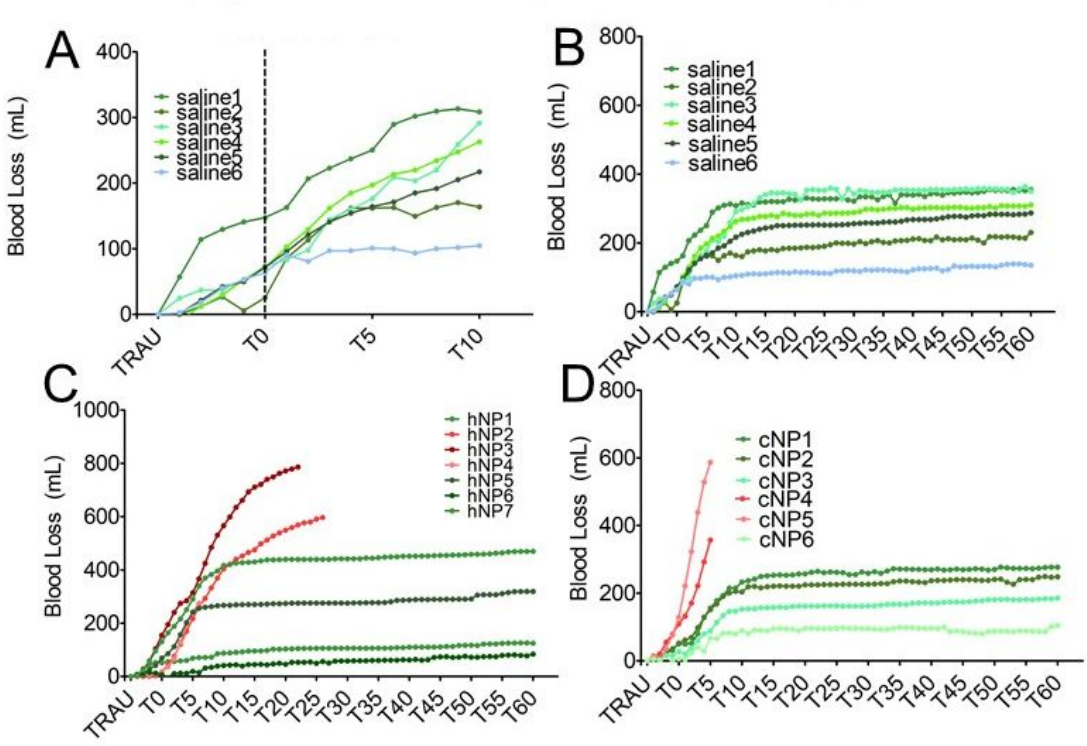
Changes in complement protein C5a in vitro

The control and treatment batches were incubated with heparinized human whole blood at 0.25mg/ml following the optimized protocol for quantifying complement protein C5a in vitro.[1] As negative control, known complement activator zymosan was used. [2] The normalized change

was determined compared to samples incubated with PBS only. The normalized change remained similar to the level observed for PBS, while zymosan lead to the highest normalized change.

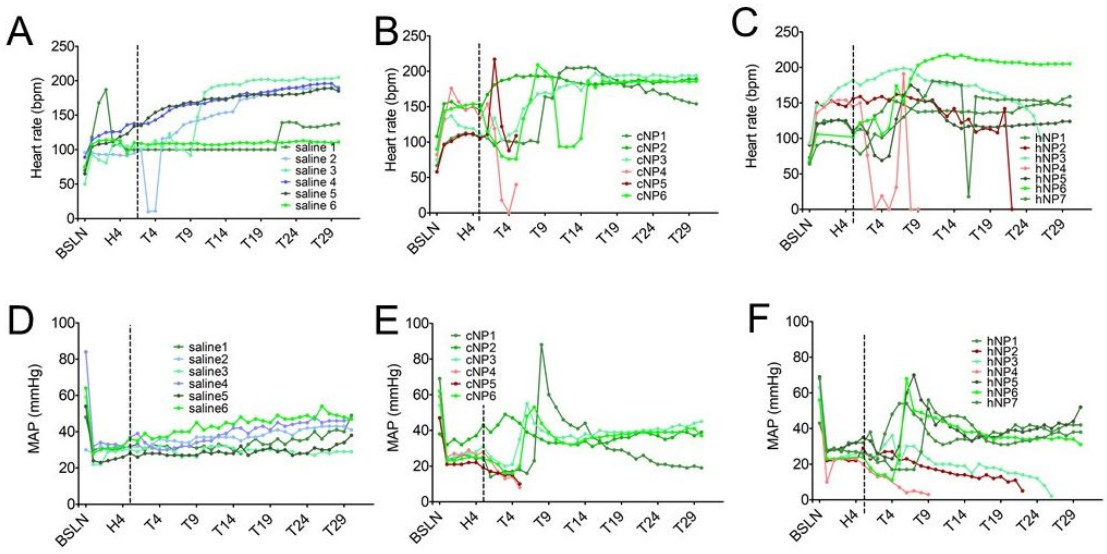


Supplementary Figure 1: Normalized change in complement protein C5a for treatment, control batches and complement activator zymosan

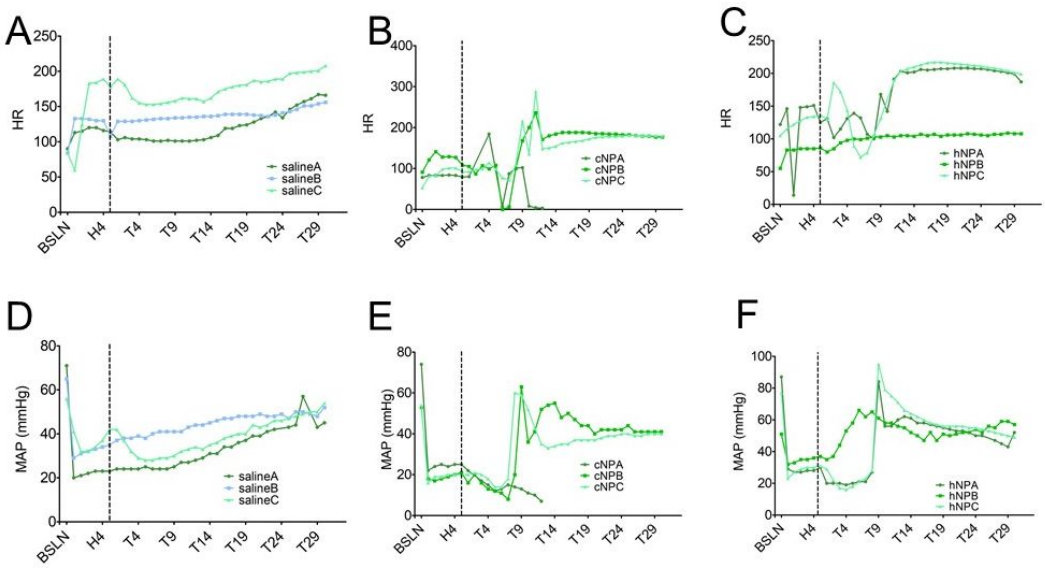


Supplementary Figure 2: Injured arm of the study (A-D). Naïve arm of the study (E). (A) Saline-treated animals showed no changes in blood loss rate following administration of saline except one (saline6) which immediately plateaued suggesting it was more likely a function of the blood

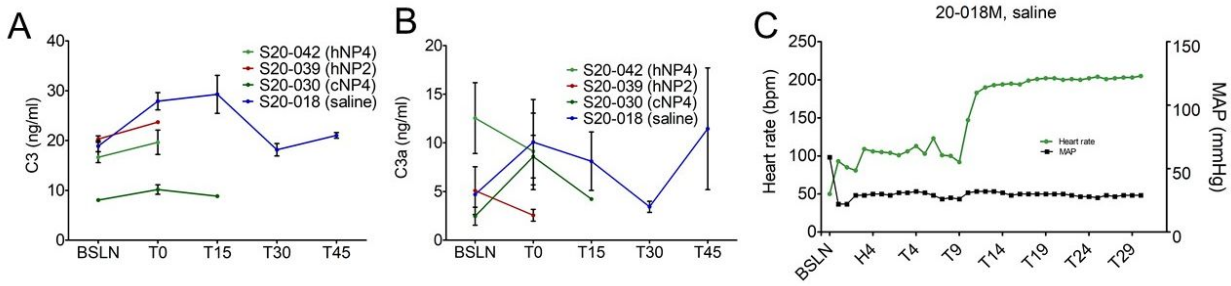
loss recording than any physiological finding. (B) Blood loss over the first 60 minutes for saline plateaued in every case. (C) 5 of the 7 animals in the hNP group had their blood loss plateau. hNP2 and hNP4 both bled out in under 30 minutes. (D) 4 of the 6 animals in the cNP group exhibited blood loss that plateaued. cNP4 and cNP65 bled out, but the rate of blood loss in both was higher before administration of the nanoparticles.



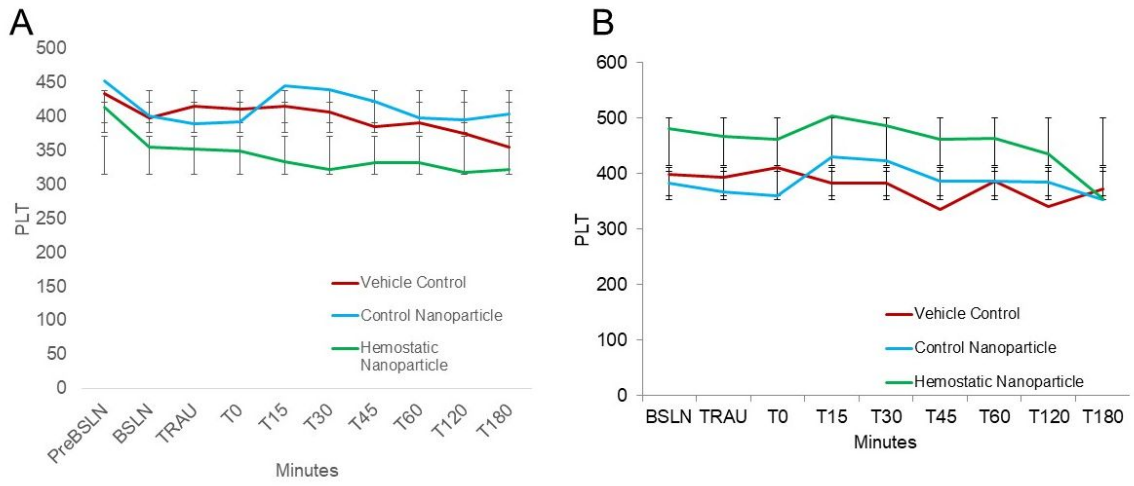
Supplementary Figure 3: Injured arm of the study. Heart rate (HR) and mean arterial pressure (MAP) for animals post treatment. (A) The HR was consistent for all of the saline-treated animals except saline 2 very briefly following saline administration. (B) In the control group, cNP4 and cNP5 show changes in HR, but whether this is due to complement activation or death is not clear. (C) hNP2 and hNP4 show changes in HR as does hNP7 which survived to the end of the experiment and showed no change in blood loss rate upon particle administration. (D) MAPs for all of the saline-treated animals was very consistent throughout the experiment. (E) MAP shows no spikes in cNP4 or cNP5 right before death but does show small spikes for a number of other cNPs. (F) MAP does not show any changes for hNP2 or hNP4 but does show small changes for other hNPs.



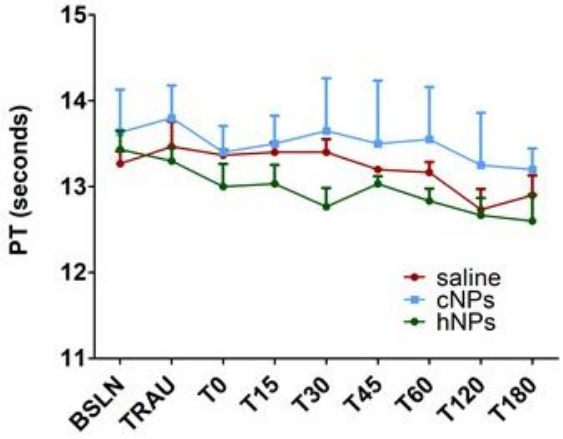
Supplementary Figure 4: Naïve arm of the study. Heart rate (HR) and mean arterial pressure (MAP) for animals post treatment in naïve animals without trauma. (A) The HR spiked for the saline-treated animals at different timepoints prior to saline administration. (B) The control group shows changes in HR around the T=7 minute timepoint. cNPA died at T=12 minutes. (C) hNPs show spikes n HR both before and after administration except hNPB. (D) MAPs for all of the saline-treated animals was consistent throughout the experiment. (E) MAP shows changes at T=7 minutes in the surviving animals. (F) MAP shows changes between T=2 minutes and 10 minutes for the hNP animals.



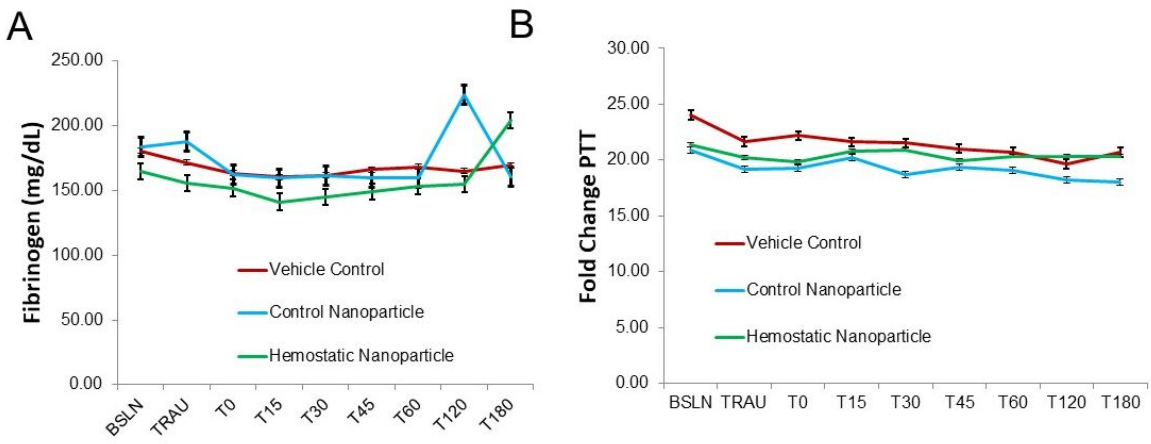
Supplementary Figure 5: Complement activation. (A) ELISA results for for C3. None of the animals with the potential for complement activation as indicated by changes in bleeding rate post particle administration show signs of complement activation by C3. Unfortunately, there is no data for T=15 for two of the animals because they died before this, but the blood draws immediately following administration at T=0 do not show rapid changes. There are greater changes seen in the saline animal which showed no signs of complement activation by any assessment. (B) ELISA results for C3a. C3a should increase if complement activation occurs. In all cases, the saline exhibits a greater, albeit tiny change suggesting there is no measurable change in complement in these animals. (C) Physiological monitoring for the saline animal further confirms there is no sign of complement activation. (error bars=SD)



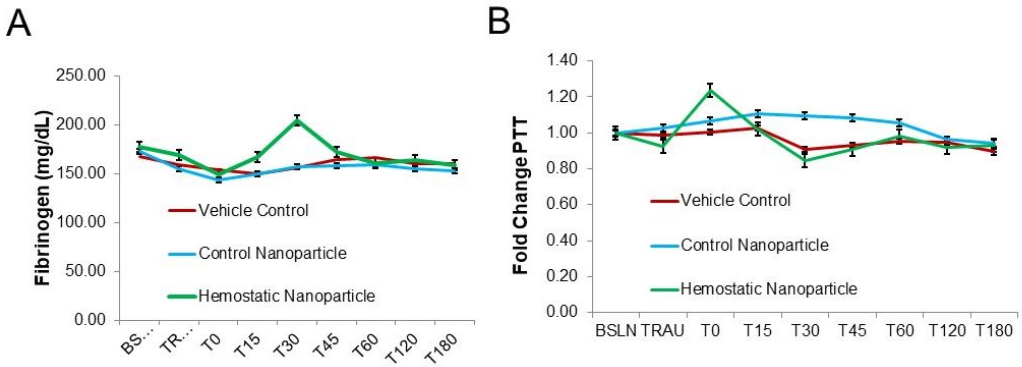
Supplementary Figure 6: Platelet counts (PLT) for the injured arm (A) and uninjured arm (B) of the study. No significant differences over time or between groups were seen. (error bars=SD)



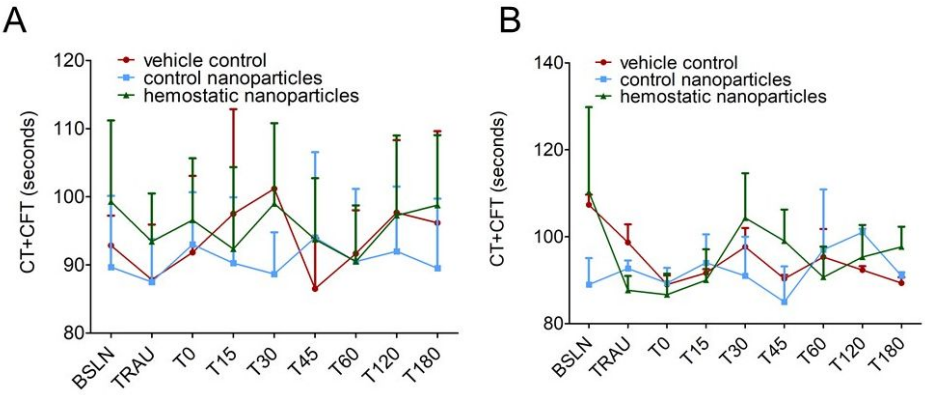
Supplementary Figure 7: The prothrombin time (PT) showed a trend to be lower for the hNP group suggesting that the hemostatic nanoparticles may reduce bleeding time in the naïve group, but the trend is not significant. (error bars=SD)



Supplementary Figure 8: No significant differences or changes were seen in the injured groups with respect to the treatment or time for either fibrinogen (A) or Partial Thromboplastin Time (PTT) (B). n=6 for each group. (error bars=SD)



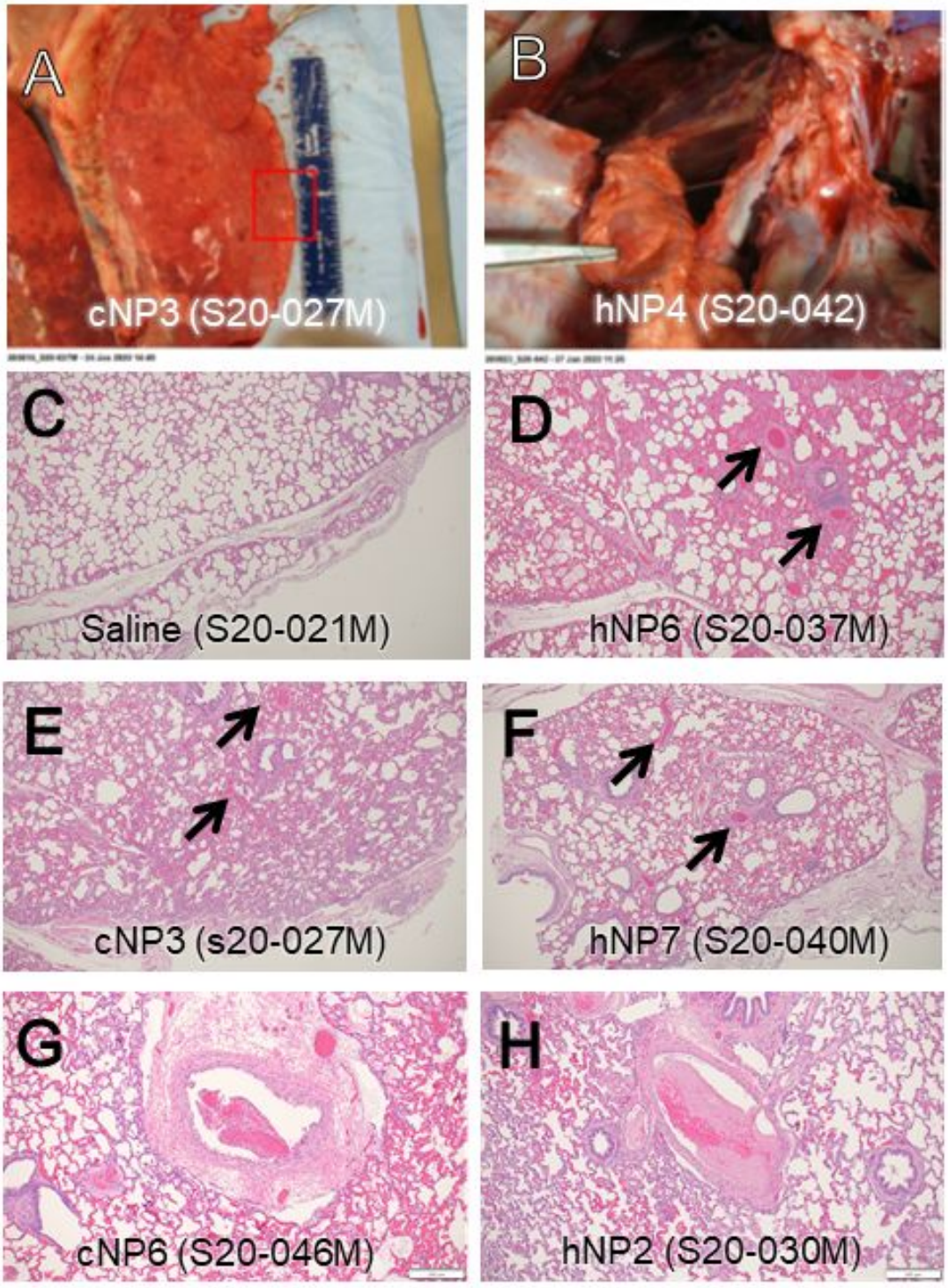
Supplementary Figure 9: Naïve arm of the study. No significant differences or changes were seen in the naive groups with respect to the treatment or time for either fibrinogen (A) or Partial Thromboplastin Time (PTT) (B). n=3 for each group. (error bars=SD)



Supplementary Figure 10: ROTEM data for (A) the injured arm of the study (n=6 per group) and the (B) naïve arm of the study (n=3). There were no significant differences or changes for the clotting time (CT+CFT) across groups or timepoints in the study. (error bars=SD)

Supplementary Table 3: Survival, Blood Loss, and Clots

Animal number	Treatment code	Treatment batch	Survival Outcome	Blood Loss (mL/kg)	Blood Loss at T=0 (ml/kg)	Clots
20-020M	saline	saline	Yes	9.92	3.12	no
20-021M	saline	saline	Yes	7.13	0.52	no
20-018M	saline	saline	Yes	7.47	1.56	no
20-026M	saline	saline	Yes	7.85	1.40	no
S20-032M	saline	saline	Yes	9.57	1.81	no
S20-034M	saline	saline	Yes	5.65	1.48	no
20-029M	cNP1	Control 14	Yes	8.75	1.09	no
20-023M	cNP2	Control 3	Yes	5.39	1.14	no
20-027M	cNP3	Control 9	Yes	5.81	0.61	yes
S20-039	cNP4	Control 1	No	10.71	2.32	no
S20-038	cNP5	Control 4	No	15.30	2.91	yes
S20-046	cNP6	Control 6	Yes	3.77	0.14	no
20-031M	hNP1	Treatment 6	Yes	6.66	1.08	no
20-030M	hNP2	Treatment 11	No	12.01	0.34	no
20-022M	hNP3	Treatment 2	No	18.08	3.43	no
S20-042M	hNP4	Treatment 8	No	9.33	0.44	yes
S20-047M	hNP5	Treatment 1	Yes	8.34	1.45	no
S20-037M	hNP6	Treatment 7	Yes	9.74	0.16	yes
S20-040M	hNP7	Treatment 12	Yes	14.55	3.37	yes



Supplementary Figure 11: Gross pathology of example clots found in the lungs along with hematoxylin and eosin (H&E) stained slides of the lungs. (A) Animal S20-027M (cNP3) exhibited clots and survived. The firm nodule is in the left middle cranial lung lobe (red box). (B) S20-042, hNP4 who exhibited clots and died. The firm nodule is on the right cranial lung lobe and is encircled by a blue circle. (C-F) Histology of left cranial lung at 40X magnification.

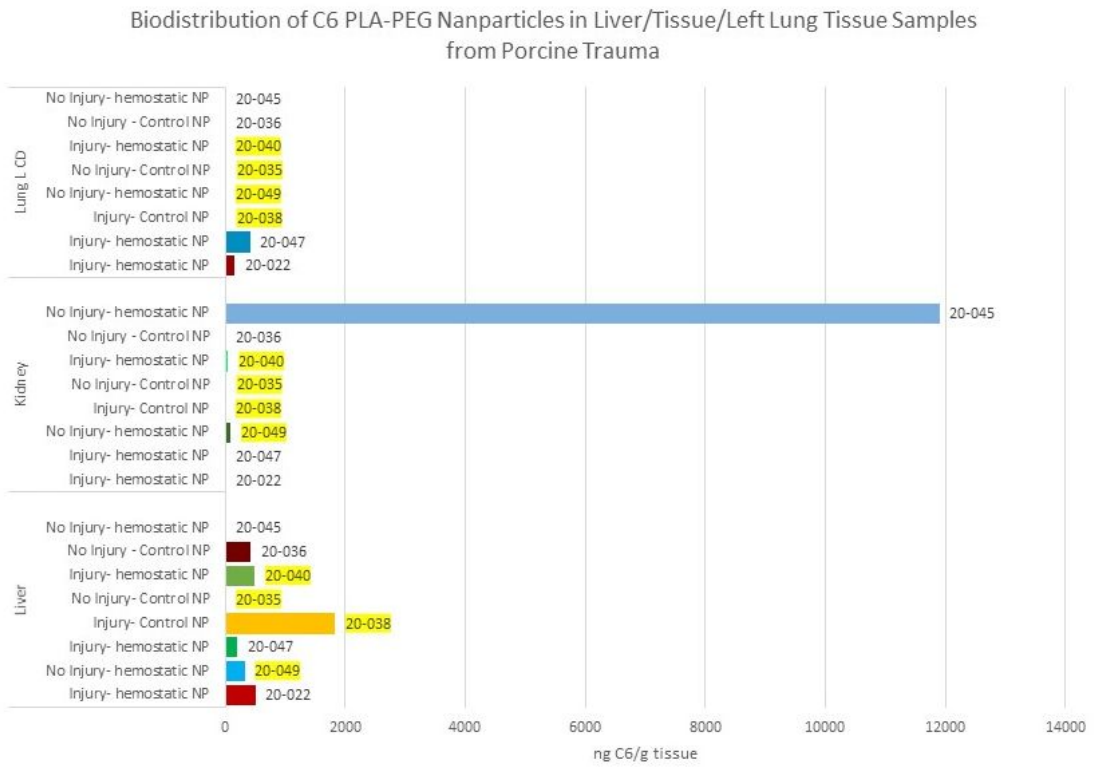
(C) Saline. Blood cells are present, but there is no evidence of clots or thrombi. (D) hNP6 (s20-037M) exhibits a number of potential thrombi. (E) Histology of cNP3 (S20-027M) exhibiting thrombi. (F) hNP7 (S20-040M) exhibiting thrombi. Example thrombi are marked by black arrows. Because of the density of thrombi in some images, not all thrombi are marked. (G) H&E of caudal lung showing a microthrombus. cNP6 (S20-046M) was not characterized as having thrombi based on the initial pathology and histological screens, but in a subsequent review at higher magnification focusing on thrombi, a microthrombus was found. (H) H&E of cranial lung of hNP2 (S20-030M) with a microthrombus at the center of the image. Thrombi are clots that are attached to vessel walls. Like cNP6, hNP2 did not exhibit the hallmarks of thrombi in the initial pathological and histological assessments, but at least one thrombus was found when screening for thrombi across all of the tissue at 100X magnification. Scale bar=200 um.

Supplementary Table 4: Survival and Clot Outcomes in the Naïve groups

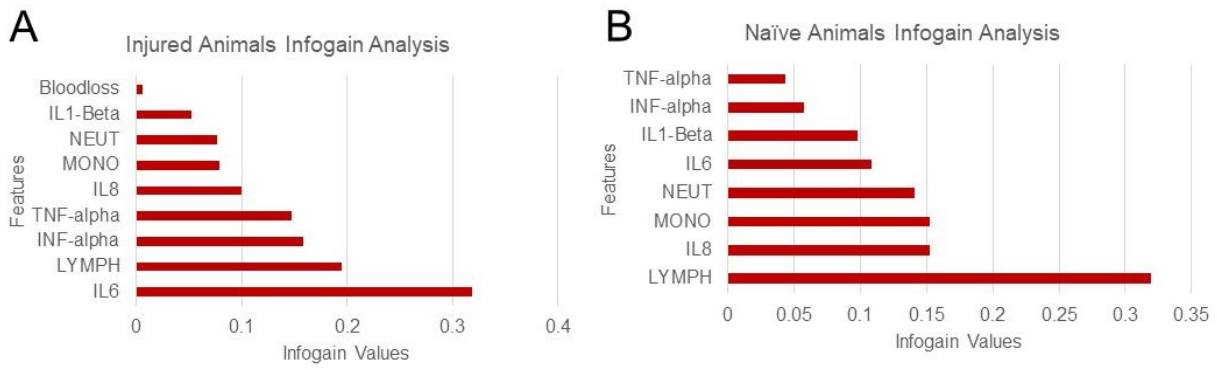
	Survival outcomes	Treatment batch	Clot outcomes
Saline control			
S20-033M	Yes	Saline	No
S20-041M	Yes	Saline	No
S20-043M	Yes	Saline	No
Control Nanoparticle			
S20-036M	No	Control 5	No
S20-035M	Yes	Control 11	Yes
S20-044M	Yes	Control 2	No
Hemostatic Nanoparticle			
S20-045M	Yes	Treatment 3	No
S20-049M	Yes	Treatment 9	Yes
S20-052M	Yes	Treatment 10	No

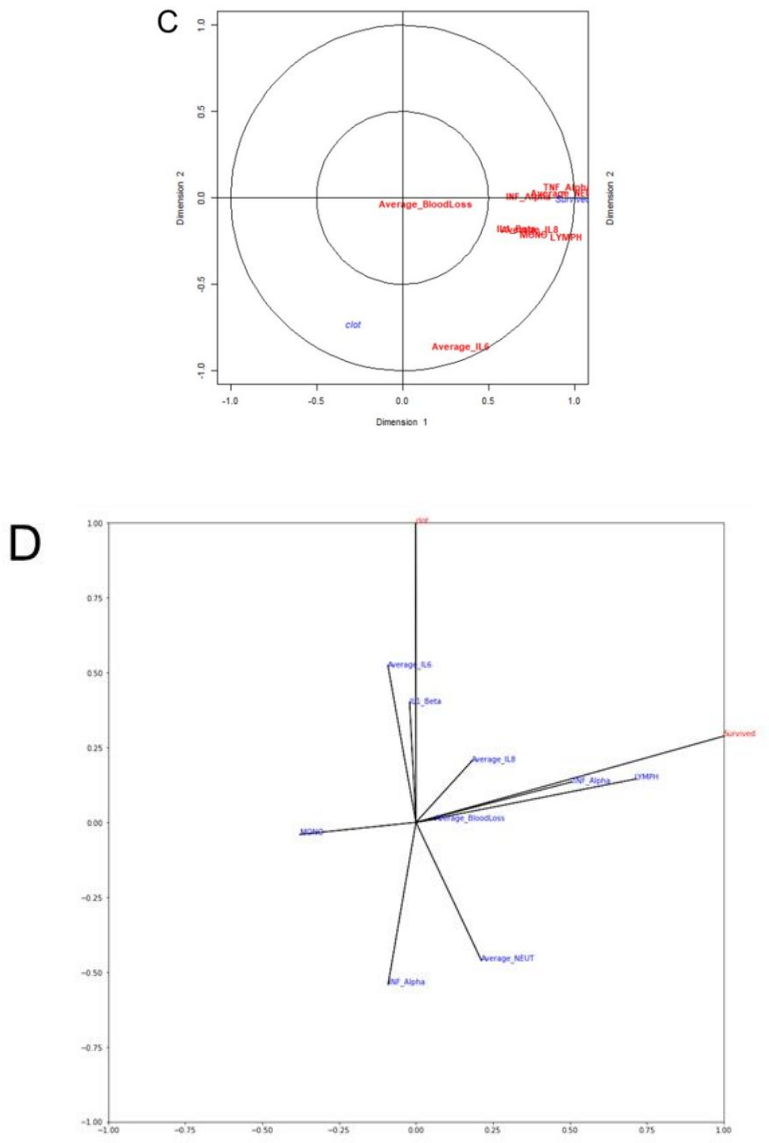
Supplementary Table 5: Size of nanoparticles as a function of outcome involving clot formation or death

Control nanoparticle Sizing (DLS)	Average	SD	Hemostatic Nanoparticle Sizing (DLS)	Average	SD
Average for all cNPs	372	40	Average for all hNPs	441	53
Average for clots	362	12	avg (clots)	456	53
Average death	405	49	average(death)	423	42

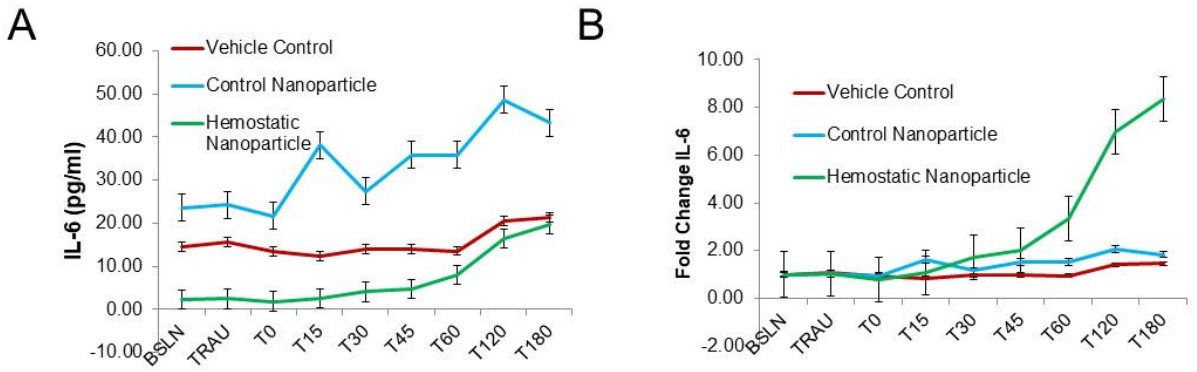


Supplementary Figure 12: Biodistribution across animals, treatments and groups. Animals that exhibited clots are highlighted in yellow. There are no significant differences between animals that had clots and animals without clots in terms of biodistribution.

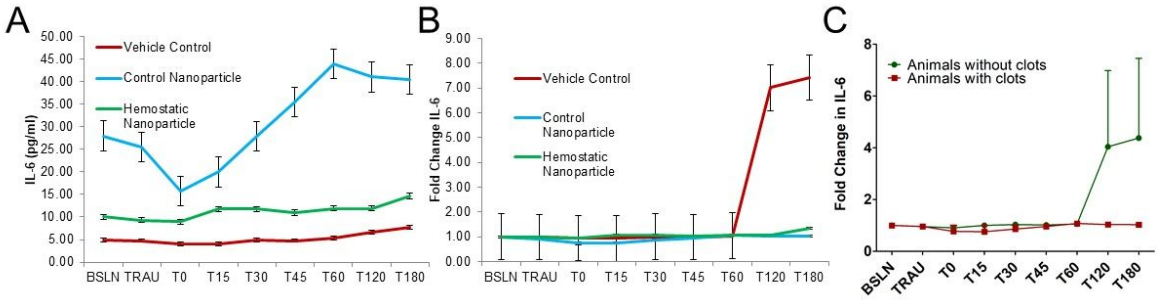




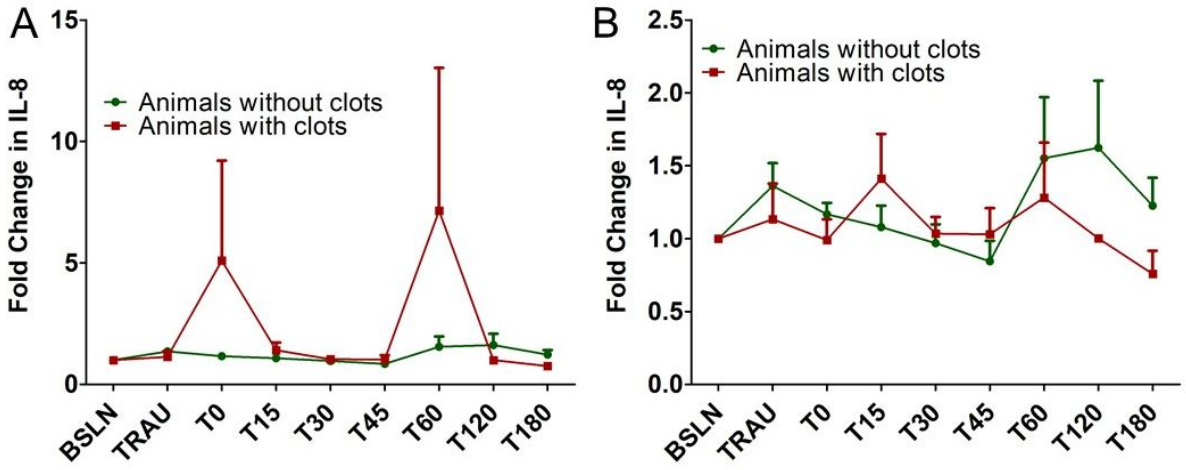
Supplementary Figure 13: Multiple data science methods were used to look for correlations between survival and parameters measured in the study (cytokines, cells, and blood loss) and clot formation. A set of features rose to the top for all the methods that correlated with both survival and clot formation. These included IL-6, TNF-alpha, neutrophils, lymphocytes, IL-8, and INF-alpha. Infogain analysis to determine important features that correlate with clot formation in the injured (A) and naïve (B) arms of the study. Importantly, similar features are important for both suggesting they correlate with the clot formation and are not artifacts of the trauma itself. (C) Canonical Correlation Analysis in R. Dimension 1 refers the independent attributes and which include blood loss, IL1, IL6, TNF-alpha while Dimension 2 refers to the dependent or target attributes which are clot and survival. (D) Canonical Correlation Analysis in Python



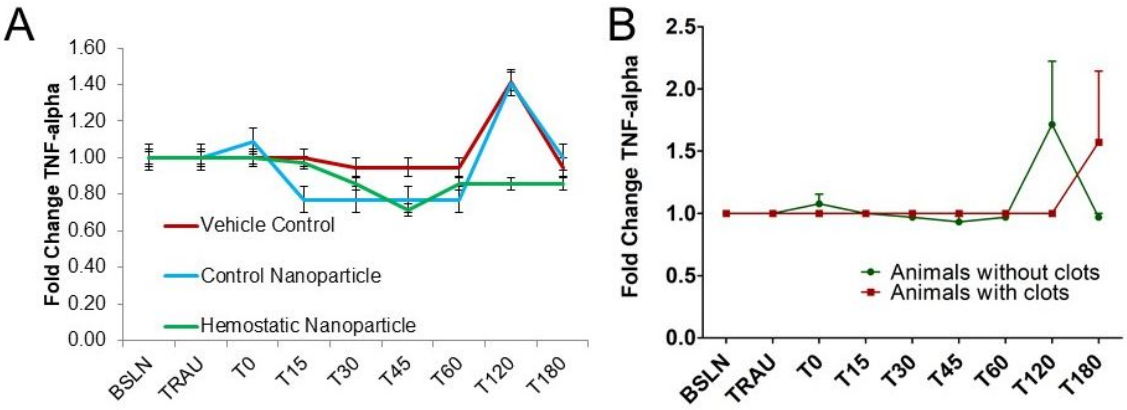
Supplementary Figure 14: (A) The concentration of IL-6 increased in all the groups over time from the baseline. (B) Normalizing the Fold change in IL-6 from baseline shows that the hemostatic nanoparticle group exhibited a greater increase in IL-6, on average, compared to either the control or vehicle groups. (error bars=SD)



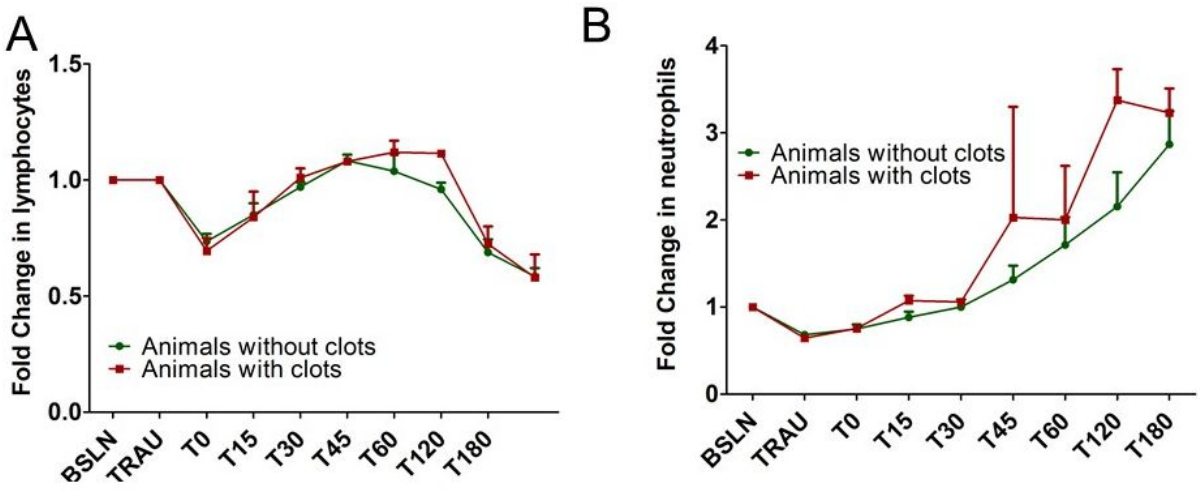
Supplementary Figure 15: Fold change in IL-6 for the naïve arm of the experiment. (A) IL-6 concentration of treatment group (n=3 per group) (B) Fold change in IL-6 for the three treatments (n=3). When the fold change is broken out by animals with clots versus animals without clots (C) The values are similar throughout with a strong outlier at the end with high changes in one animal. (7 animals without clots and 2 with clots in the naïve group) (error bars=SD)



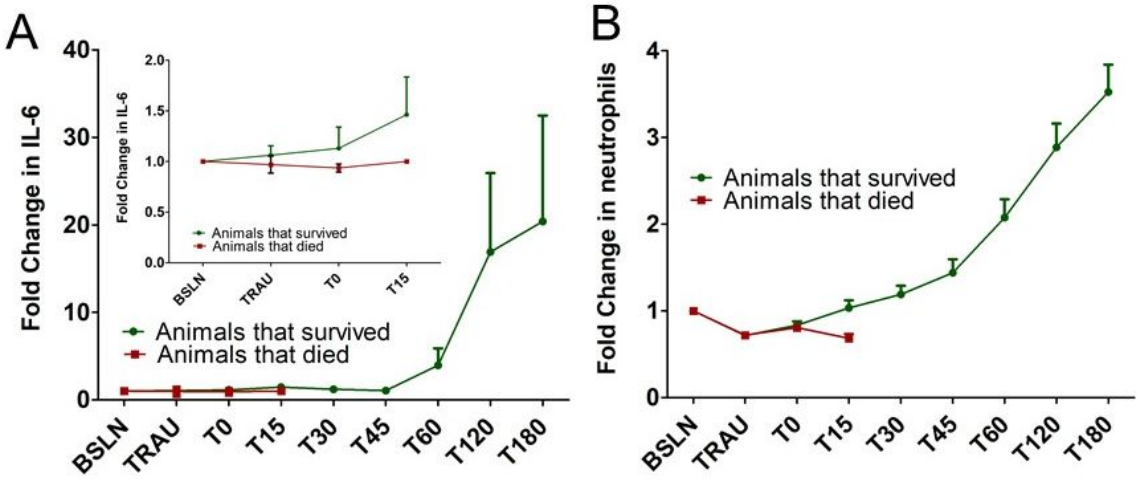
Supplementary Figure 16: Fold change in IL-8. (A) Fold change in IL-8 broken out by animals with clots versus those without. There were two timepoints for one animal that were many fold higher than all other values. (B) To resolve the finer resolution data, the one animal was removed and the y axis expanded. The fold change in IL-8 does not follow a particular pattern that would, in and of itself, provide clear trends that can be correlated with outcomes. (error bars=SD)



Supplementary Figure 17: Fold change in TNF-alpha. (A) There are no significant differences between groups for TNF-alpha. Likewise, there are no significant differences between animals with clots and animals without clots for fold change in TNF-alpha (B). There is a trend to animals without clots showing a reduction in TNF-alpha during the first 60 minutes post administration, but the difference is very small. (error bars=SD)



Supplementary Figure 18: (A) There are no differences in the changes with lymphocytes for animals with (n=2) and without clots (n=7). (B) Neutrophils are slightly elevated in the animals with clot s(n=2) compared to the animals without clots (n=7) but the differences are not significant in this small data set. (error bars=SD)



Supplementary Figure 19: (A) IL-6 increases for animals that survive following treatment compared to those that died. It should be noted that at T=15 only 2 of the 5 animals that died were still alive and contribute data to these timepoints. (B) Likewise, neutrophils increase for animals that survived compared to those that died. In both cases, the number of animals that died is small (n=5) and the two that were alive at T=15 minutes died shortly after the 15 minute timepoint. (error bars=SD)

References

1. Maisha, N.; Coombs, T.; Lavik, E., Development of a Sensitive Assay to Screen Nanoparticles in Vitro for Complement Activation. *ACS Biomaterials Science & Engineering* **2020,** *6* (9), 4903-4915.
2. Keystone, E.; Schorlemmer, H.; Pope, C.; Allison, A., Zymosan—induced arthritis. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **1977,** *20* (7), 1396-1401.