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Supplementary Materials for

Neutrophil extracellular traps exacerbate neurological deficits after traumatic brain injury

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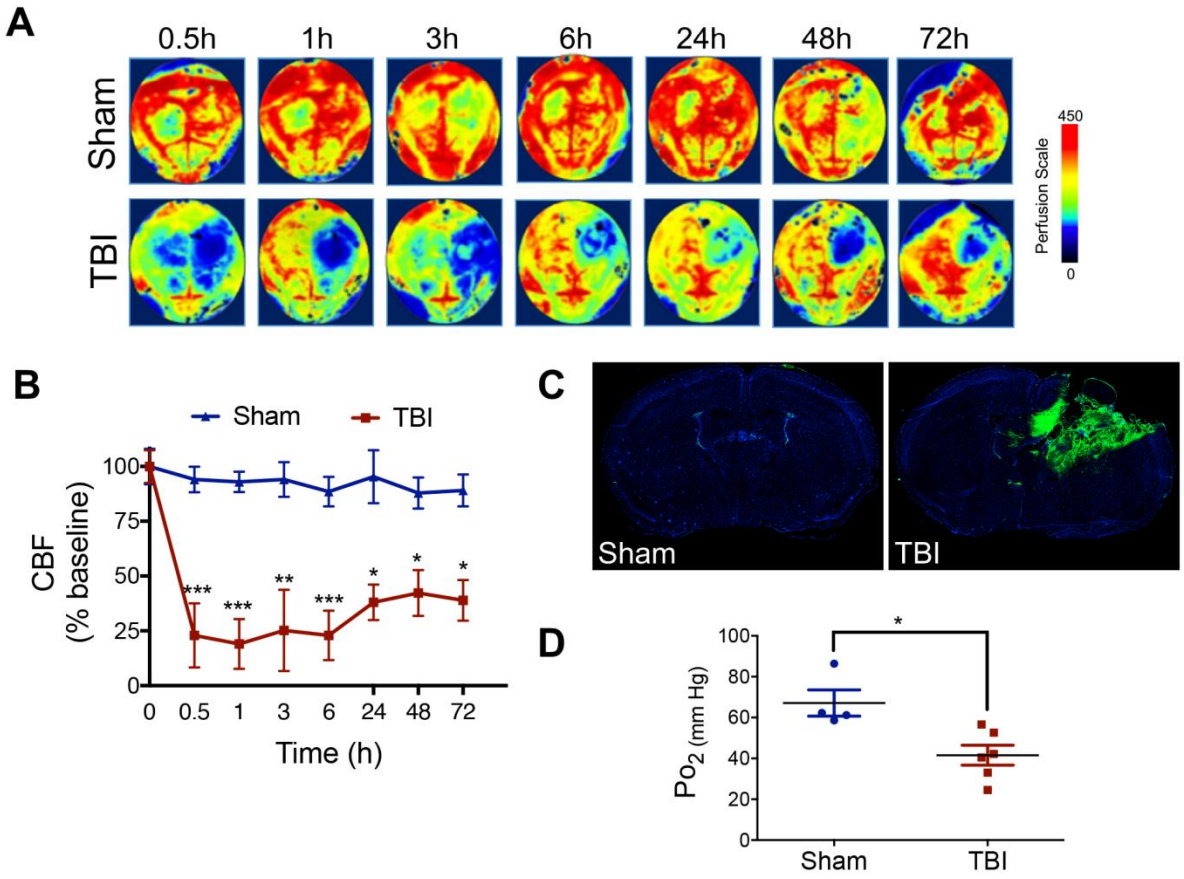
This PDF file includes:

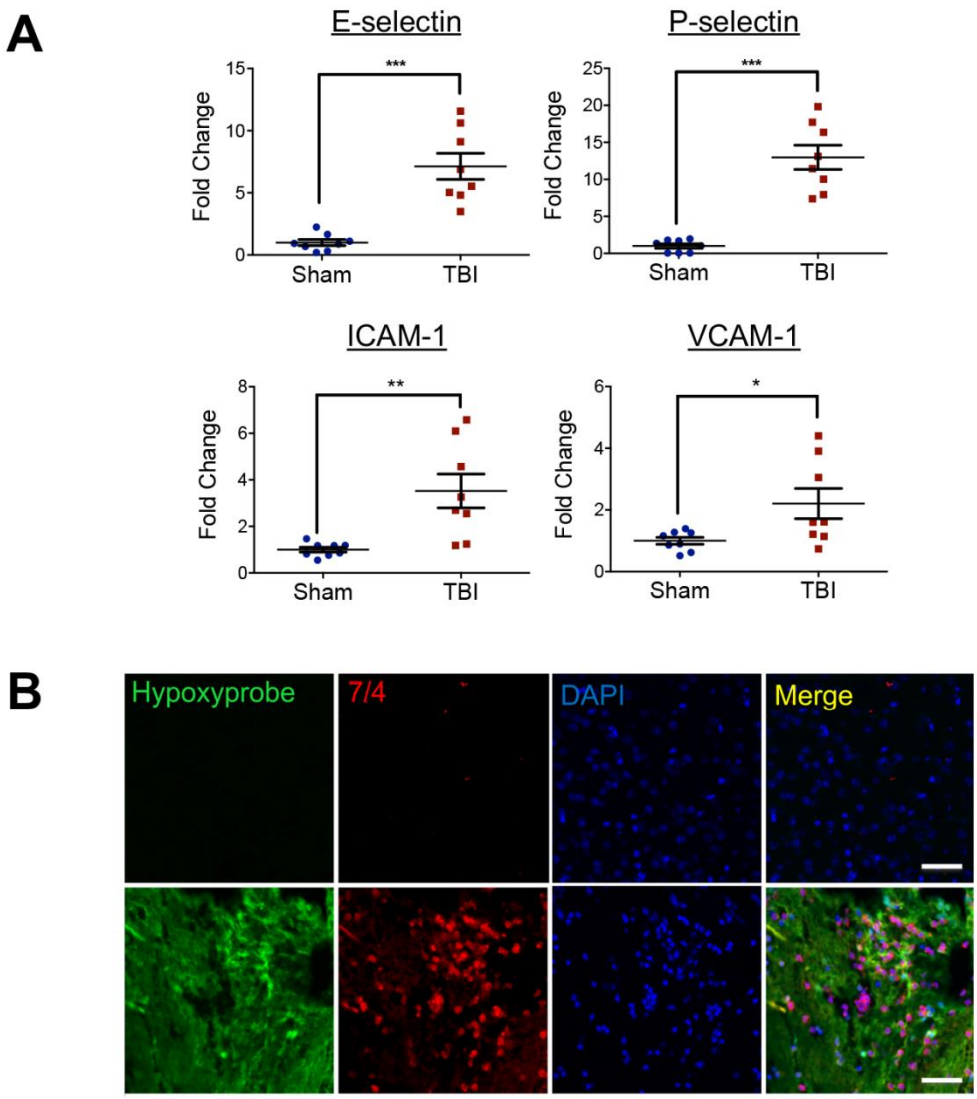
SUPPLEMENTARY MATERIALS

Supplemental Figure 1:



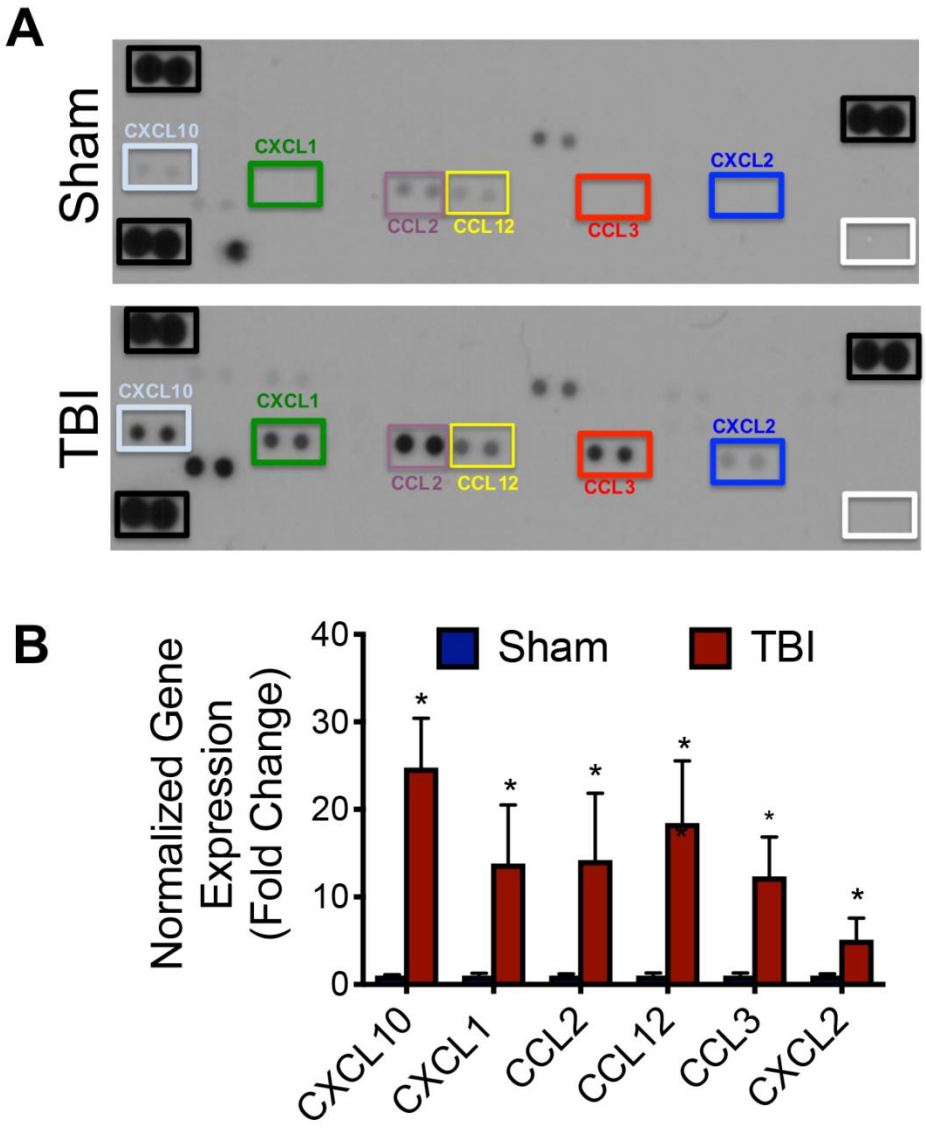
Supplemental Fig. 1. **Temporal changes in cerebral hypoperfusion after TBI.** (**A, B**) Mice were subjected to sham injury or TBI and CBF was quantified using laser speckle contrast imaging (LSCI). Representative images are provided for each time point (0.5-72h) and reflect an immediate and sustained reduction in cerebral perfusion over the first 72h post-TBI. Data are expressed as mean ± SEM (n=8 mice/group) from two independent experiments and were analyzed within each time point by Student's t-test (*p<0.05, **p<0.01, ***p<0.001 vs. sham). (**C**) Mice were injected with 50 mg/kg pimoidazole HCl (Hypoxyprobe), which binds with hypoxic tissue (<10 mM Hg), prior to tissue collection at 3h post-sham/TBI. Pimonidazole adducts (green) were visualized in coronal brain sections using a Keyence BZ-X700 microscope. DAPI was used as a counterstain. Hypoxic tissue was observed throughout the peri-contusional cortex, but not sham brain, spatially overlapping with the region of cerebral hypoperfusion. Representative micrographs are provided from n=6 mice/group. (**D**) Intraparenchymal partial pressure of oxygen (pO_2) was determined in isoflurane-anesthetized mice using a 50 μm Unisense O_2 microsensor at 24h post-sham/TBI. Brain tissue pO_2 is expressed as mean ± SEM from 4-6 mice/group and analyzed using a Student's t-test (*p=0.012 vs. control). Data was replicated in two independent experiments.

Supplemental Figure 2:



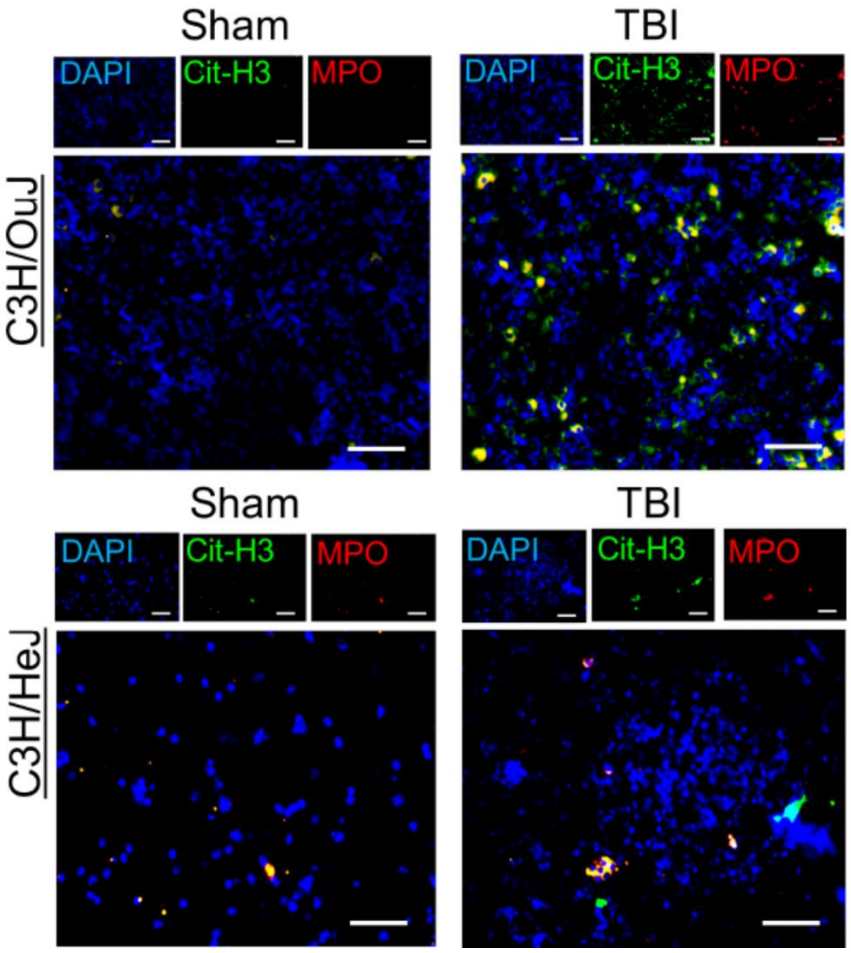
Supplemental Fig. 2. **Increased neutrophil trafficking into hypoxic tissue after TBI. (A)** qRT-PCR analysis of mRNA isolated from peri-contusional cortex or anatomically matched sham brain tissue at 24h post-injury revealed a TBI-induced increase in the expression of E-selectin ($p<0.001$), P-selectin ($p<0.001$), ICAM-1 ($p<0.01$), and VCAM-1 ($p<0.05$), as compared to sham. Data are expressed as mean ± SEM from n=8 mice/group. (**B**) Mice were injected with 50 mg/kg pimoidazole HCl (Hypoxyprobe) and fixed brain tissue was prepared for immunohistochemistry at 3h post-sham/TBI. Pimonidazole adducts (green) co-localized with neutrophil marker 7/4 in the peri-contusional cortex, as assessed by confocal microscopy. DAPI was used as a counterstain. Data are representative of n=6 mice/group. Scale bar = 50 μm.

Supplemental Figure 3:



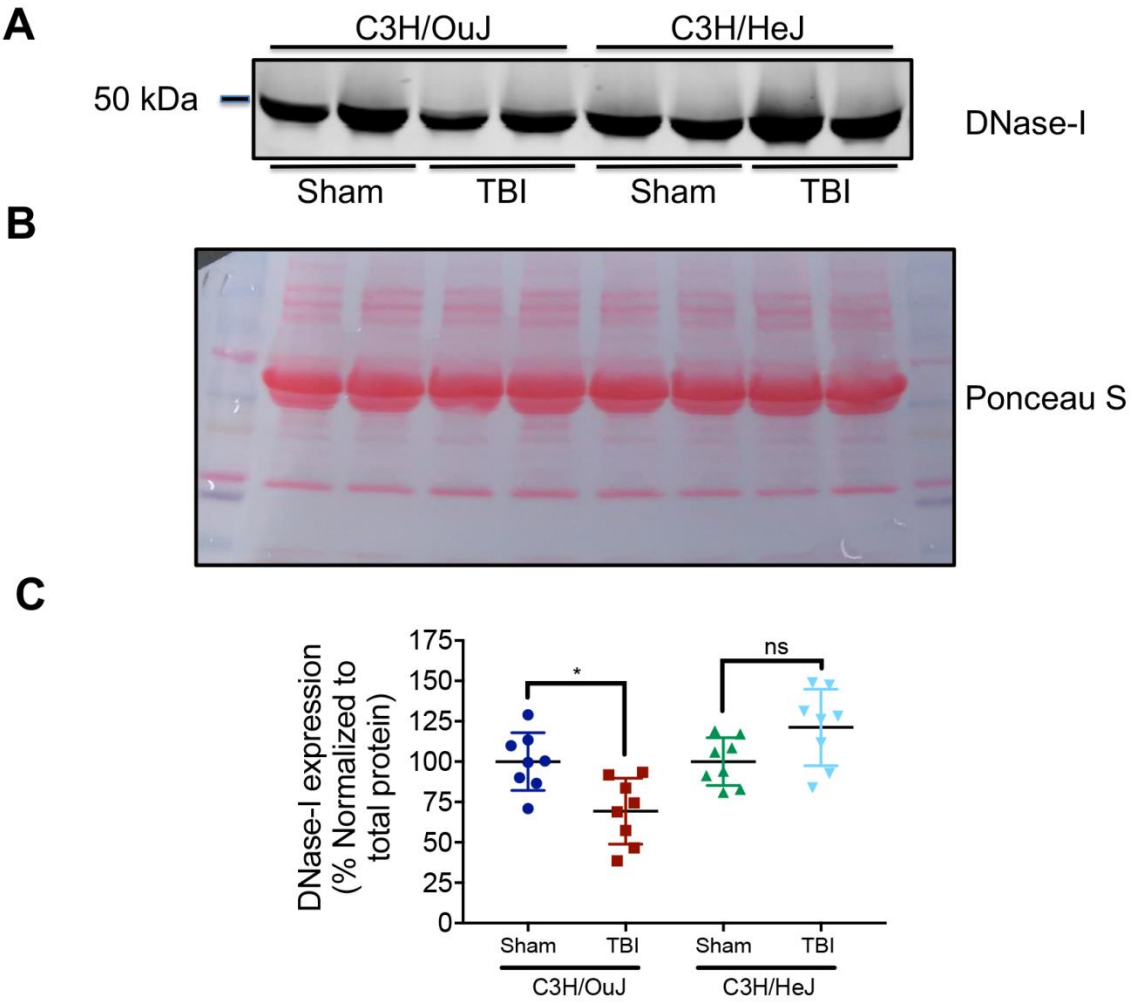
Supplemental Fig. 3. **Increased expression of leukocyte chemoattractants after TBI. (A)** Protein lysates from peri-contusional cortex (or anatomically matched brain tissue from sham mice) was collected at 24h post-injury, pooled (n=3 mice/sample), and screened using a R&D Systems Mouse Cytokine Array Panel A. Proteins exhibiting the largest increases after TBI included CXCL10, CXCL1, CCL2, CCL12, CCL3, and CXCL2. Black boxes are reference spots and the white box is a negative control. Experiments and arrays were performed in duplicate. **(B)** mRNA was isolated from peri-contusional cortex or anatomically matched sham brain tissue and used for qRT-PCR to validate changes observed in the protein array. Normalized gene expression data are expressed as fold change versus sham from n=6 mice/group. Data were analyzed by a Student's t-test and expressed as mean ± SEM (* $p<0.05$ vs. sham).

Supplemental Figure 4:



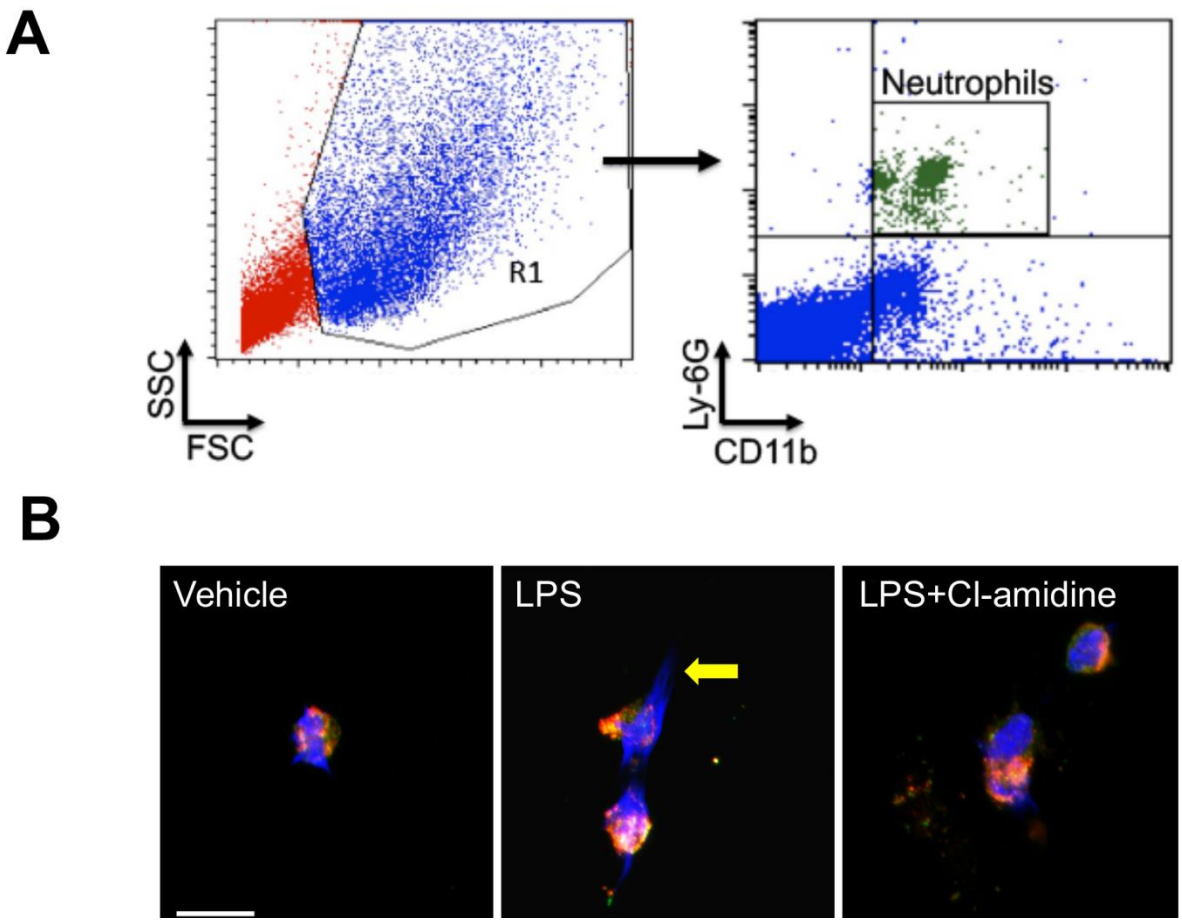
Supplemental Fig. 4. **TLR4-dependent NET formation within brain after TBI.** Single cell suspensions were prepared from brain tissue of wild-type (C3H/OuJ) or TLR4 mutant (C3H/HeJ) mice at 24h post-sham/TBI. Neutrophils ($CD11b^+$, $Ly\text{-}6G^+$, $Gr\text{-}1^+$) were sorted by magnetic bead isolation and re-suspended in PBS at 20,000 cells/300 µL. Sorted neutrophils were next adhered to glass microscope slides with the aid of a cytocentrifuge and then stained with DAPI (blue; chromatin), Cit-H3 (green), and MPO (red). Yellow color in the merged images (larger panels) identifies the presence of extracellular NETs. Data are representative of three independent experiments. Scale bar = 100 µm.

Supplemental Figure 5:



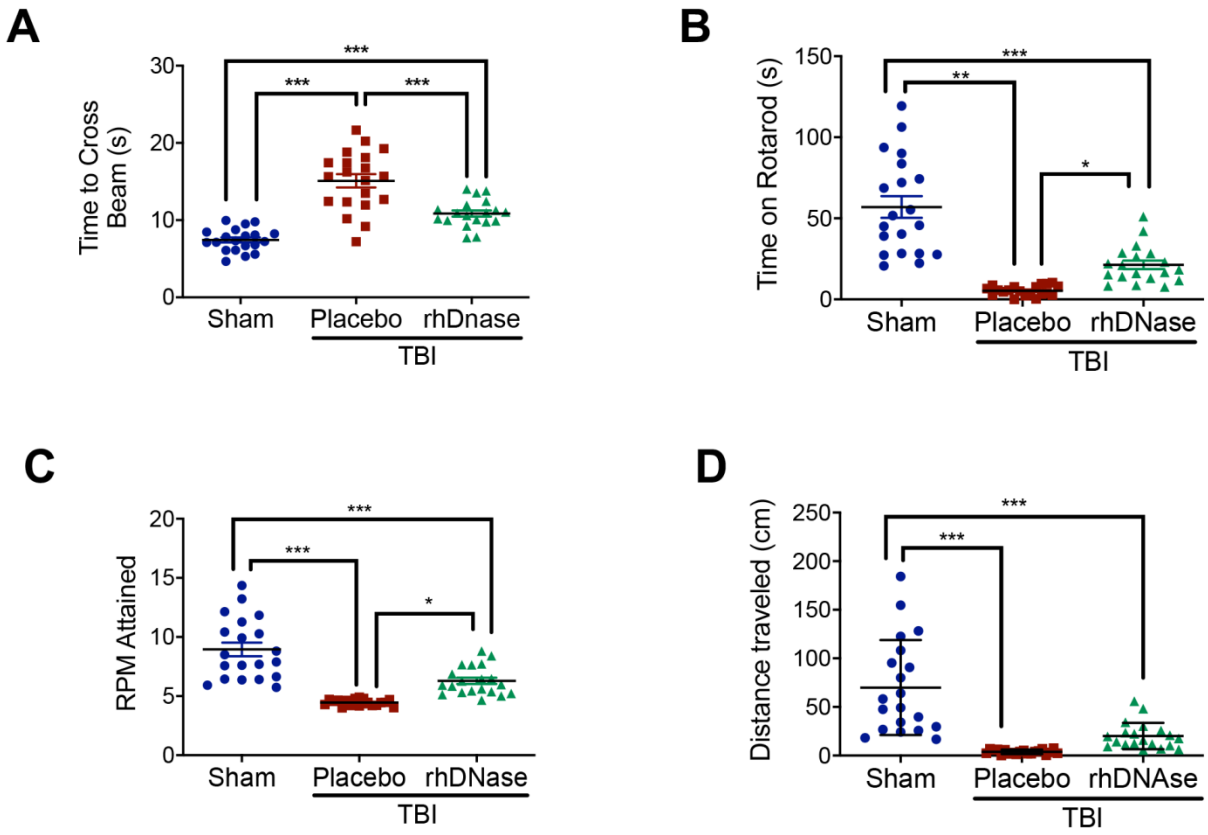
Supplemental Fig. 5. **TLR4-dependent reduction in serum DNase-I after TBI. (A)** Protein lysates (20 μg) were prepared from the serum of C3H/OuJ or C3H/HeJ mice at 24 hours post-sham/TBI. Western blots were probed for DNase-1. (**B**) Protein loading was analyzed by Ponceau S staining. (**C**) Densitometry analysis revealed the reduced expression of serum DNase-I expression in C3H/OuJ mice after TBI, as compared to sham-operated controls. C3H/HeJ mice exhibited no differences in serum DNase-I expression after TBI. Data are representative of n=8 mice/group and were analyzed using a Student's t-test within each genotype (*$p<0.05$, ns = not statistically significant). Data are expressed as mean ± SEM.

Supplemental Figure 6:



Supplemental Fig. 6. **PAD4 mediates TLR4-induced NET formation. (A)** Mouse neutrophils (>99% purity) were isolated from the spleen of a naïve mouse using a MoFlow XDP cell sorter (top panel), plated on coverslips, and treated for 3h with vehicle, lipopolysaccharide (LPS; 100 ng/mL), or LPS+50 μM Cl-amidine. **(B)** Fixed cells were stained with anti-Cit-H3 (red; neutrophils) and DAPI (blue; a chromatin label). Note the disrupted nuclear morphology and thread-like appearance of chromatin after LPS treatment (see arrow) was reversed by co-treatment with Cl-amidine. Scale bar = 10 μm.

Supplemental Figure 7:



Supplemental Fig. 7. rhDNase improves acute neurological outcomes after TBI. Administration of rhDNase (5 mg/kg, i.v.) at 1h post-TBI improved neurological function, including a reduction in the time required to traverse a narrow beam and improved motor function (time spent on rotarod, RPM attained, and distance traveled on rotarod) on an accelerating rotarod. Data are mean ± SEM from n=20 mice/group and were analyzed by One-Way ANOVA followed by Tukey's post-hoc test (*p<0.05, **p<0.01, ***p<0.001).