TriService Nursing Research Program Final Report Cover Page

Sponsoring Institution TriService Nursing Research Program

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Bethesda MD 20814

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Title of Research Study: Iron Activation of Cellular Oxidases: Modulation of Neuronal Viability (In Vitro).

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Applicant Organization: Henry Jackson Foundation for the Advancement of Military Medicine

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Purpose: Traumatic brain injury (TBI) is a significant health issue with 361,092 service members diagnosed. Increased iron, oxidative stress and microglia are present within TBI lesions acutely after injury. Microglia are major modulators of the inflammatory response and produce neurotoxic reactive oxygen species (ROS) via NADPH oxidases (NOX). We suspected that iron is a major contributor to neuro-degeneration by contributing to oxidative stress through microglia. We hypothesized that iron utilized NOX derived ROS to accentuate ROS synthesis in activated microglia and reduced neuronal survivability *in vitro*.

Design: Experimental design that utilized immortalized BV2 microglia, 8 pregnant Sprague-Dawley rats for primary fetal mixed neuronal cultures, and PC12's neuronal like cells.

Methods: BV2 microglia were exposed to ferrous sulfate, lipopolysaccharide, GSK2795039 or GKT137831 and then subsequently co-cultured with neurons or PC12s. Co-culture conditions were maintained for 24 hours and cells were harvested for ROS detection, protein quantification, gene expression, and morphologic alterations by immunocytochemistry.

Sample: Immortalized and primary rodent cell cultures

Analysis: Iron dose dependently amplified ROS production among lipopolysaccharide activated microglia. This increased ROS did not modulate gene or protein expression of microglial polarization markers or cytokine/chemokine production. It exacerbated neurotoxicity among primary neurons and differentiated PC12s. NOX2 or NOX4 inhibition significantly reversed irons accentuation of ROS production and subsequently ameliorated neurotoxicity.

Findings: This data substantiates iron's contributions to oxidative stress within TBI and provides mechanistic insight into NOX2 and NOX4 contributions to this pathology. This research illustrates the importance of iron mitigation or NOX inhibition as possible future treatment modalities for TBI.

Implications for Military Nursing: This project establishes a foundation for future research and may significantly influence nursing practice through modification of blood product administration protocols. Our novel research suggested a potential therapeutic advantage by free iron scavenging therapy, antioxidant administration, or NOX inhibition in the presence of iron.

TSNRP Research Priorities that Study or Project Addresses

Primary Priority

Force Health Protection:	☐ Fit and ready force ☒ Deploy with and care for the warrior ☐ Care for all entrusted to our care
Nursing Competencies and Practice:	☐ Patient outcomes ☐ Quality and safety ☐ Translate research into practice/evidence-based practice ☐ Clinical excellence ☐ Knowledge management ☐ Education and training
Leadership, Ethics, and Mentoring:	☐ Health policy ☐ Recruitment and retention ☐ Preparing tomorrow's leaders ☐ Care of the caregiver
Other:	☐

Progress Towards Achievement of Specific Aims of the Study or Project

Findings related to each specific aim of the study or project, answering each research or study questions, and/or hypothesis:

The experimentation over the first year determined that exposure of activated BV2 microglia to free iron, *in vitro*, resulted in an increased presence of reactive oxygen species (ROS). We extensively investigated this phenomenon to complete my first research aim: the characterization of free iron exposure to microglia. We discovered a complex relationship exists between free iron and microglial polarization.

The experimentation over the second year focused on the impact and contributions of NOX2 and NOX4 enzymes to the iron exacerbated oxidative stress mechanism, which represents our second aim. Finally, we conducted co-culture experimentation between primary neurons or immortalize neuronal-like PC12 cultures with iron exposed activated microglia. We present data that suggests our original hypothesis, **Ferrous iron (Fe^{2+}) utilizes NOX2 and NOX4 derived ROS to accentuate a pro-inflammatory M1 Phenotype in microglia, which reduces neuronal viability *in vitro***, is partially correct in that polarization may not be directly affected by iron but neuronal toxicity and ultimate survivability is reduced.

Aim
1. Characterize the response of microglia to iron *in vitro*.

Intracellular iron detected 24 hours following treatment.

First, we determined if ferrous sulfate (FeSO4) was present intracellularly within the cell cultures. To accomplish this task we selected the presence of Divalent Metal Transporter 1 (DMT1) and the gene expression of Ferritin (FER) as an indirect measure of irons presence. The presence of DMT1

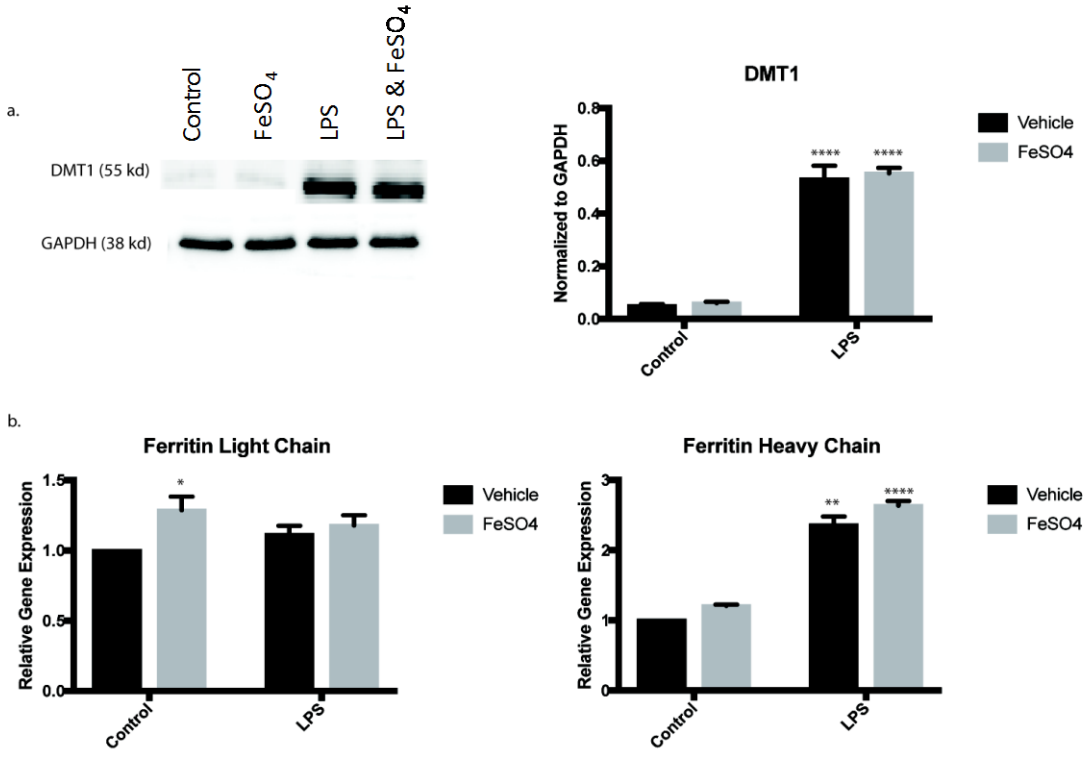

Figure 1. BV2 express both DMT1 and light & heavy chain ferritin. a) Western blotting of DMT1 revealed increased expression of those groups treated with LPS. b) Gene expression of light and heavy chain ferritin displayed a significant increase in light chain ferritin within $FeSO_4$ groups. Heavy chain ferritin revealed a more robust response to LPS with groups exposed to LPS increasing gene expression. All groups were compared using two-way ANOVA with Tukey post-test. N = 4/group. *p<0.05, **p<0.01, ****p<0.0001. Bars represent mean +/- SEM.

ensured BV2 cultures possessed the cellular machinery necessary for iron transport as described in other microglial cell line literature. Cell cultures were treated and harvested according to protocols described in the methods section of the grant. We utilized Western blotting to determine the relative protein concentration present at 24 hours after treatment. We detected the presence of DMT1 in all treatment groups suggesting that all treatment groups possess the machinery necessary to transport free iron. Light and heavy chain ferritin mRNA were isolated, amplified, and quantified by real time-polymerase chain reaction (RT-PCR) methods to determine gene expression. All data were normalized to control and significant differences were detected among groups treated with iron alone in regards to light chain ferritin (Control vs. 100 μM $FeSO_4$, $p<0.0467$). Although lipopolysaccharide (LPS) treated groups with and without iron were not significantly different from controls in light chain ferritin expression, among heavy chain ferritin it significantly increased in response to LPS (Control vs. LPS, $p<0.0001$), (Control vs. LPS & 100 μM $FeSO_4$, $p<0.0001$). As expected, heavy chain ferritin, the catalytic enzyme of ferritin responsible for converting Fe^{2+} to Fe^{3+}, increased in gene expression after LPS exposure.

Iron independently induced ROS production and accentuated LPS derived ROS synthesis.

In order to determine oxidative stress among the treatment groups we determined the presence of reactive oxygen species with a 2′,7′-dichlorofluorescin diacetate assay. We conducted a dose response curve with the Fe^{2+} donor, ferrous sulfate ($FeSO_4$), to determine ROS concentrations. We plated cells into groups with 0 (control), 10, 25, 50, 100 μM of $FeSO_4$ with and without LPS. We detected a significant dose response increase of ROS among the groups treated with 100 μM $FeSO_4$ (Ctrl vs 100 μM $FeSO_4$, $p<0.0047$). In addition, LPS induced the production of ROS alone (Ctrl vs. LPS, $p<0.0023$) and throughout all groups treated with both LPS and increasing concentrations of $FeSO_4$ in an incremental dose response pattern (LPS vs: LPS & 10 μM $FeSO_4$, $p<0.0001$; LPS & 25 μM $FeSO_4$, $p<0.0001$; LPS & 50 μM $FeSO_4$, $p<0.0001$; LPS & 100 μM $FeSO_4$, $p<0.0001$). (Figure 2a)

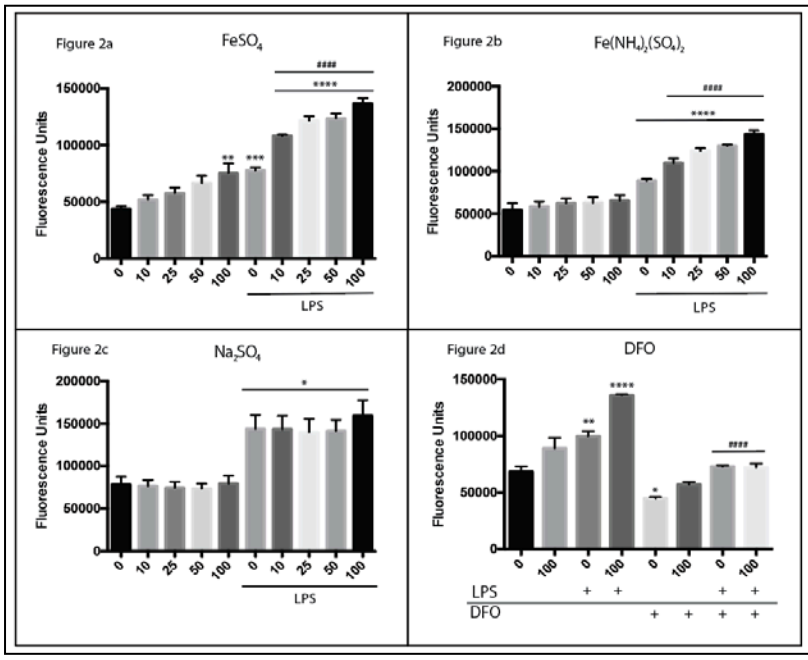

Figure 2. Iron exacerbates ROS generation independently and accentuates LPS induced ROS production among microglia. Numbers on the X axis represent micro molar concentrations of FeSO4, Fe(NH4)2(SO4)2, or Na2SO4. (Figure 2a) FeSO4 exposure produced a dose response rise ROS when compared between control vs. 100 μM FeSO4 and all groups treated with LPS. Significant differences were detected between LPS and all groups treated with both FeSO4 and LPS. Fe(NH4)2(SO4)2 exposure produced similar effects as FeSO4 among the cultures. In addition, significant ROS increases were noted among all groups treated with LPS when compared with control. (Figure 2b) LPS and Fe(NH4)2(SO4)2 simultaneous exposure produced increased concentrations of ROS. (Figure 2c) Na2SO4 did not produce an incremental patterned increase of ROS as previously described. LPS treated groups did produce an increased amount of ROS, although no differences were noted between the groups treated with LPS. (Figure 2d) We treated groups with either 100 μM concentration of FeSO4 or with LPS alone and together which increased ROS production significantly. The addition of 250 μM concentrations of DFO reduced ROS concentrations to control levels. N=3 in all groups. All groups were compared using one-way ANOVA with tukeys post hoc test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, and ####p<0.0001. All data presented in SEM.

To ensure this phenomenon was not unique to $FeSO_4$, we replaced $FeSO_4$ with another Fe^{2+} donor, ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$). We detected a similar increased ROS accentuation pattern within the groups treated with both LPS and $Fe(NH_4)_2(SO_4)_2$ (Ctrl vs. LPS,

p<0.0001), (LPS vs: LPS & 10 μM $FeSO_4$, p<0.0001; LPS & 25 μM $FeSO_4$, p<0.0001; LPS & 50 μM $FeSO_4$, p<0.0001; LPS & 100 μM $FeSO_4$, p<0.0001). (Figure 2b)

Next, we ruled out the influence of the sulfate groups within the treatments. We exposed the cultures to $NaSO_4$ instead of the $FeSO_4$. We determined that the all groups with LPS treatment induced a significant increase in ROS production (Ctrl vs. LPS, p<0.0001), (LPS vs: LPS & 10 μM $FeSO_4$, p<0.0001; LPS & 25 μM $FeSO_4$, p<0.0001; LPS & 50 μM $FeSO_4$, p<0.0001; LPS & 100 μM $FeSO_4$, p<0.0001). However, there were no differences between the LPS treated groups suggesting that the sulfate group contributions to the ROS phenomenon were negligible. (Figure 2c)

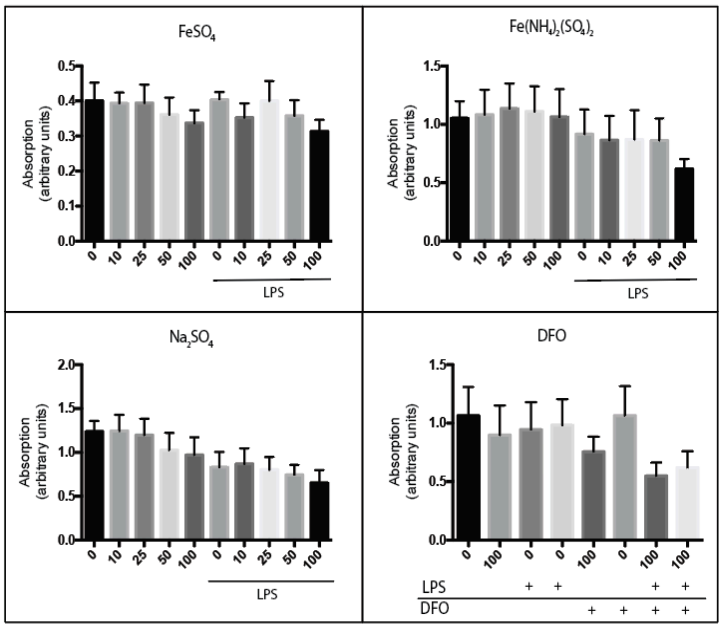

Figure 3. No signficant differences were noted in LDH between the groups treated with various compounds and LPS. N=3. All data presented in SEM

In order to ensure the iron is contributing to the accentuation of ROS production, we incorporated deferoxamine (DFO), an effective iron chelator, into the groups. First, we treated the cells to LPS, $FeSO_4$, or both as previously described and then added DFO at a concentration of 250 μM. The cells responded to LPS and $FeSO_4$ exposure by accentuating ROS production (Ctrl vs: $FeSO_4$, p < 0.06; LPS, p < 0.002; $FeSO_4$ & LPS, p < 0.0001). The addition of DFO significantly ameliorated the ROS accentuation by reducing ROS levels among all groups to control levels (Ctrl vs DFO, p < 0.242); ($FeSO_4$ vs. DFO & $FeSO_4$, p<0.0016); (LPS vs. DFO & LPS, p<0.0084); and ($FeSO_4$ & LPS vs. DFO & $FeSO_4$ & LPS, p<0.0001).

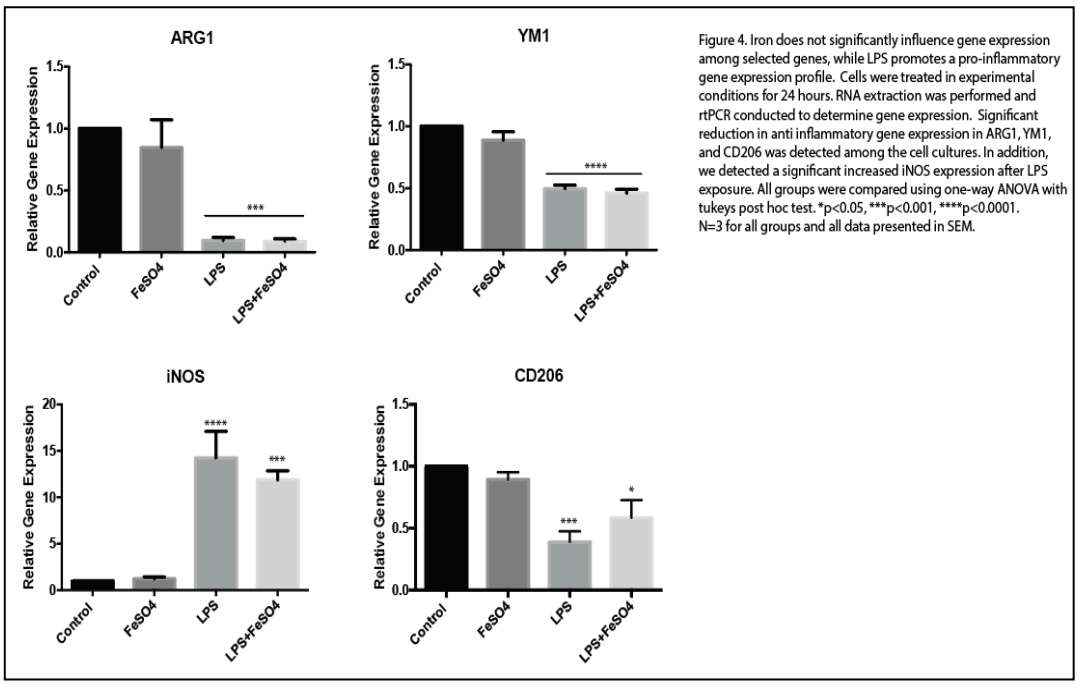

Figure 4. Iron does not significantly influence gene expression among selected genes, while LPS promotes a pro-inflammatory gene expression profile. Cells were treated in experimental conditions for 24 hours. RNA extraction was performed and rtPCR conducted to determine gene expression. Significant reduction in anti inflammatory gene expression in ARG1, YM1, and CD206 was detected among the cell cultures. In addition, we detected a significant increased iNOS expression after LPS exposure. All groups were compared using one-way ANOVA with tukeys post hoc test. *p<0.05, ***p<0.001, ****p<0.0001. N=3 for all groups and all data presented in SEM.

(Figure 2d)

As control experiments, concurrent LDH assays of media taken from treatment groups revealed no significant differences between groups were detected suggesting that cellular death among the cultures did not contribute to the increase of ROS. Also in order to ensure the increase in ROS was not due to proliferation of the cells within the treatment groups we utilized a BIORAD TC20 to calculate a cell count among each group. No significant differences were noted between the groups.

Iron alone does not encourage a pro-inflammatory polarization alterations

To determine polarization phenotype after exposure to $FeSO_4$ and LPS, we evaluated the gene expression profile through the intracellular concentrations of mRNA of arginase-1 (ARG1), chitinase-like enzyme (YM1), inducible nitric oxide synthase (iNOS) and CD206 at 24 hours after treatment. In all markers, the addition of LPS induced an overall reduction in anti-inflammatory markers and an increase in pro-inflammatory markers: ARG1 (Ctrl vs: LPS, $p<0.0002$; $FeSO_4$ & LPS, $p<0.0002$), YM1 (Ctrl vs: LPS, $p<0.0001$; $FeSO_4$ & LPS, $p<0.0001$), CD206 (Ctrl vs: LPS, $p<0.0009$; $FeSO_4$ & LPS, $p<0.0206$), iNOS (Ctrl vs: LPS, $p<0.0001$; $FeSO_4$ & LPS, $p<0.0006$). $FeSO_4$ alone did not significantly influence gene expression within any group (Figure 4).

Next, we determined polarization profiles through fluorescent activated cell sorter (FACS). We harvested treated groups at 24 hours and prepared the cells for FACS. Interestingly, We detected an over abundance of CD86 and CD16/32 basal expression among the BV2

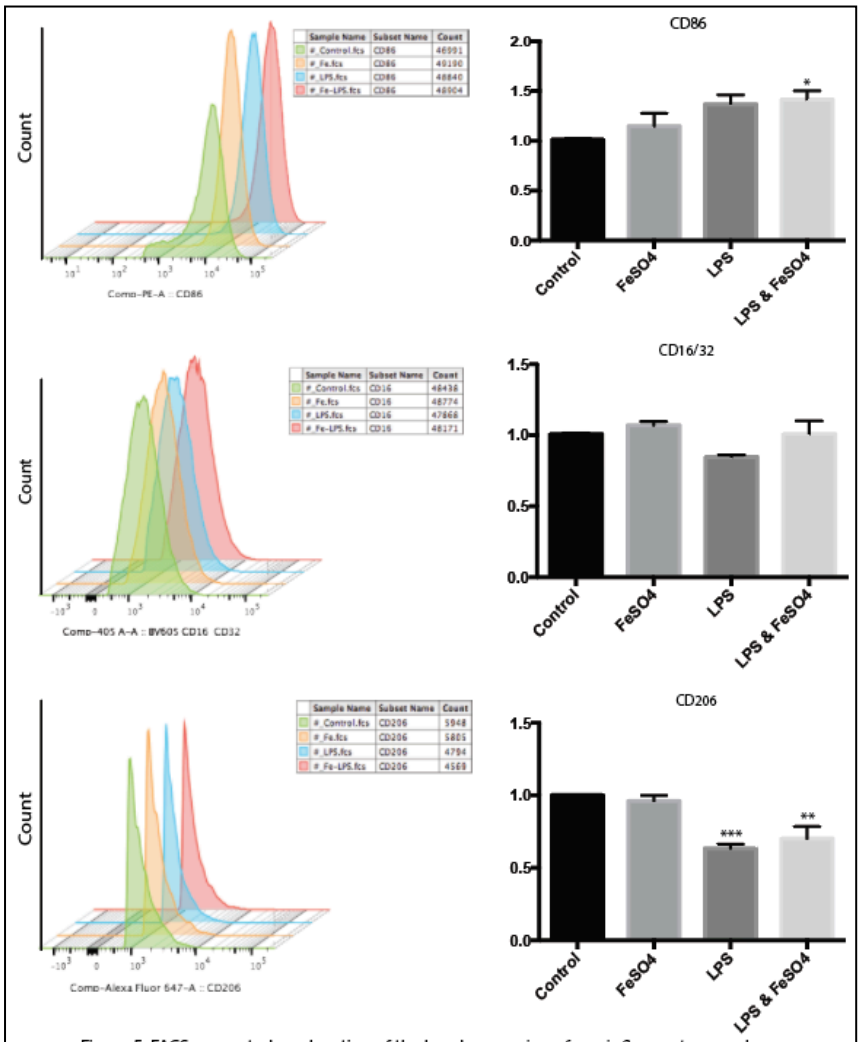

Figure 5. FACS suggested an elevation of the basal expression of pro-inflammatory markers among microglia. However, iron does not induce an alteration in the chosen markers despite production of ROS. Overlays illustrate fluorescence distribution among microglia after 24 hour exposure to experimental conditions. All groups were compared using one-way ANOVA with tukey post hoc test. *p<0.05, **p<0.01, ***p<0.001. N=3 and presented in SEM.

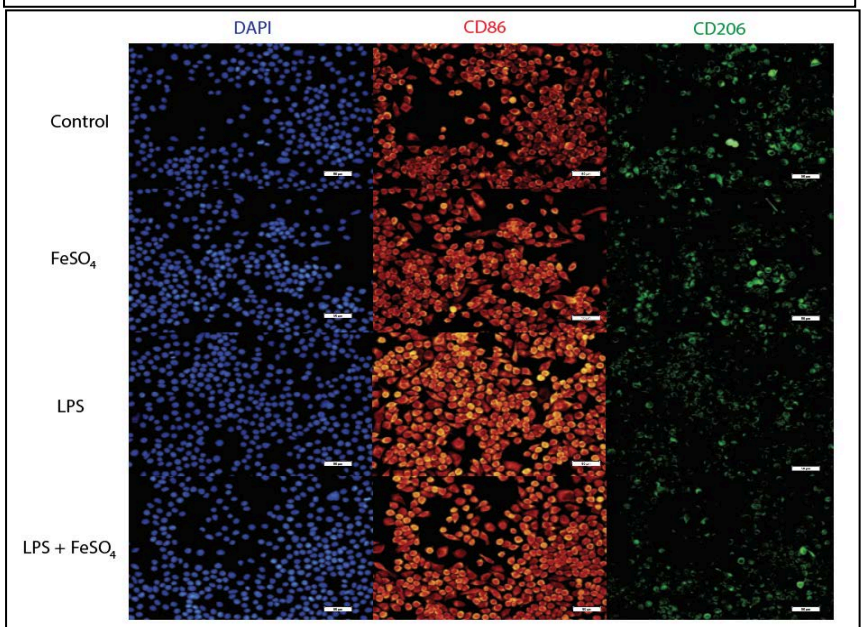

Figure 6. Qualitative immunocytochemistry supports FACS and gene expression data suggesting elevated basal expression of CD86 among microglia. We detected an increased expression of CD86 and decrease of CD206 among the groups treated with LPS. Iron did not influence CC86 and CD206 at 24 hours. Representative sample presented.

microglia. After normalizing all groups to control, we found the intensity of CD86 staining among groups treated with LPS alone or concurrently with $FeSO_4$ increased significantly (Ctrl vs. LPS, $p<0.0738$; Ctrl vs. $FeSO_4$ & LPS, $p<0.0402$). In addition, we detected a significant reduction in CD206 fluorescence staining among the groups (Ctrl vs. LPS, $p<0.0009$; Ctrl vs. $FeSO_4$ & LPS, $p<0.0047$). No significant changes to CD16/32 staining were noted among the groups.

To corroborate FACS data we employed immunocytochemistry to determine protein expression of CD86 and CD206, two well known polarization markers. We determined LPS induced a reduction in CD206 while increasing pro-inflammatory CD86 expression among microglia, which substantiated the conclusions from the FACS.

Iron does not significantly affect of cytokine or chemokine production 24 hours after exposure.

To determine physiologic behavior of the cultures after treatment, we utilized a proteome profiler to determine cytokine and chemokine production among the treatment groups. Although we screened for 30 different proteins, we detected alterations in the cytokines and chemokines listed in Table 1. Overall, LPS continues to have an overwhelming effect among the cells by promoting pro-inflammatory cytokine or chemokine synthesis: IL-1rα ($FeSO_4$ vs. LPS, $p<0.054$; $FeSO_4$ vs. $FeSO_4$ & LPS, $p<0.043$); IL-1α (Ctrl vs. LPS, $p<0.0055$; Ctrl vs. $FeSO_4$ & LPS, $p<0.0074$); IL-1β (Ctrl vs. LPS, $p<0.006$; Ctrl vs. $FeSO_4$ & LPS, $p<0.0007$); IP-10 (Ctrl vs. LPS, $p<0.0053$; Ctrl vs. $FeSO_4$ & LPS, $p<0.0058$); RANTES (Ctrl vs. LPS, $p<0.0006$; Ctrl vs. $FeSO_4$ & LPS, $p<0.0002$); TNFα (Ctrl vs. LPS, $p<0.0017$; Ctrl vs. $FeSO_4$ & LPS, $p<0.005$) (Figure 7). Interestingly, iron does not seem to have an independent effect on the markers selected within the profiler assay.

	Control		FeSO4		LPS		LPS & FeSO4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G-CSF	ND	NA	ND	NA	0.074	0.022	0.067	0.013
sICAM-1	0.690	0.440	0.813	0.555	0.813	0.561	0.698	0.451
IL-1a	0.171	0.031	0.179	0.036	0.486	0.120	0.470	0.092
IL-1β	ND	NA	ND	NA	0.183	0.039	0.177	0.046
IL-1ra	0.934	0.091	0.870	0.102	1.165	0.040	1.180	0.180
IP-10	0.175	0.073	0.143	0.046	0.365	0.018	0.362	0.036
M-CSF	0.097	0.017	0.107	0.009	0.085	0.007	0.083	0.018
JE	0.573	0.087	0.535	0.095	0.619	0.085	0.568	0.107
MIP-1α	0.941	0.028	1.070	0.200	0.849	0.111	0.769	0.033
MIP-1β	0.034	0.002	0.031	0.017	0.113	0.048	0.106	0.041
MIP-2	ND	NA	ND	NA	0.072	0.017	0.072	0.004
RANTES	0.079	0.008	0.081	0.015	0.186	0.032	0.206	0.014
SDF-1	0.039	0.013	0.038	0.012	0.040	0.015	0.036	0.011
TIMP-1	0.052	0.001	0.057	0.017	0.058	0.005	0.063	0.005
TNFα	0.067	0.008	0.074	0.019	0.241	0.066	0.214	0.025
TREM-1	0.080	0.003	0.080	0.012	0.103	0.006	0.091	0.014

Table 1. Listing of detected chemokines and cytokines from Proteome Profiler. N=3. All data presented in SD.

Iron increases toxicity among neurons during co-culture. After determining the polarization profile of cells treated with $FeSO_4$ with and without LPS, we evaluated the effect of treated microglia on neurons. Co-culture experimentation utilized a predominately-neuronal mixed primary rat culture, a generous gift from Dr. Byrnes, with BV2 exposure through transwells as previously described in methods. Immunocytochemistry revealed a toxic effect on neurons by microglia treated with LPS alone or LPS with $FeSO_4$ (Figure 8).

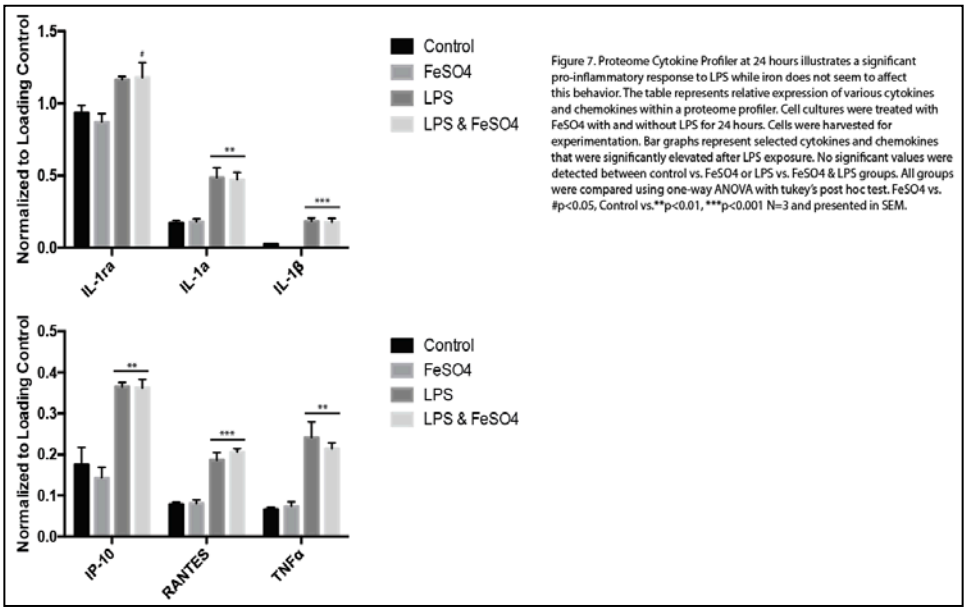

Figure 7. Proteome Cytokine Profiler at 24 hours illustrates a significant pro-inflammatory response to LPS while iron does not seem to affect this behavior. The table represents relative expression of various cytokines and chemokines within a proteome profiler. Cell cultures were treated with FeSO4 with and without LPS for 24 hours. Cells were harvested for experimentation. Bar graphs represent selected cytokines and chemokines that were significantly elevated after LPS exposure. No significant values were detected between control vs. FeSO4 or LPS vs. FeSO4 & LPS groups. All groups were compared using one-way ANOVA with tukey's post hoc test. FeSO4 vs. #p<0.05, Control vs.**p<0.01, ***p<0.001 N=3 and presented in SEM.

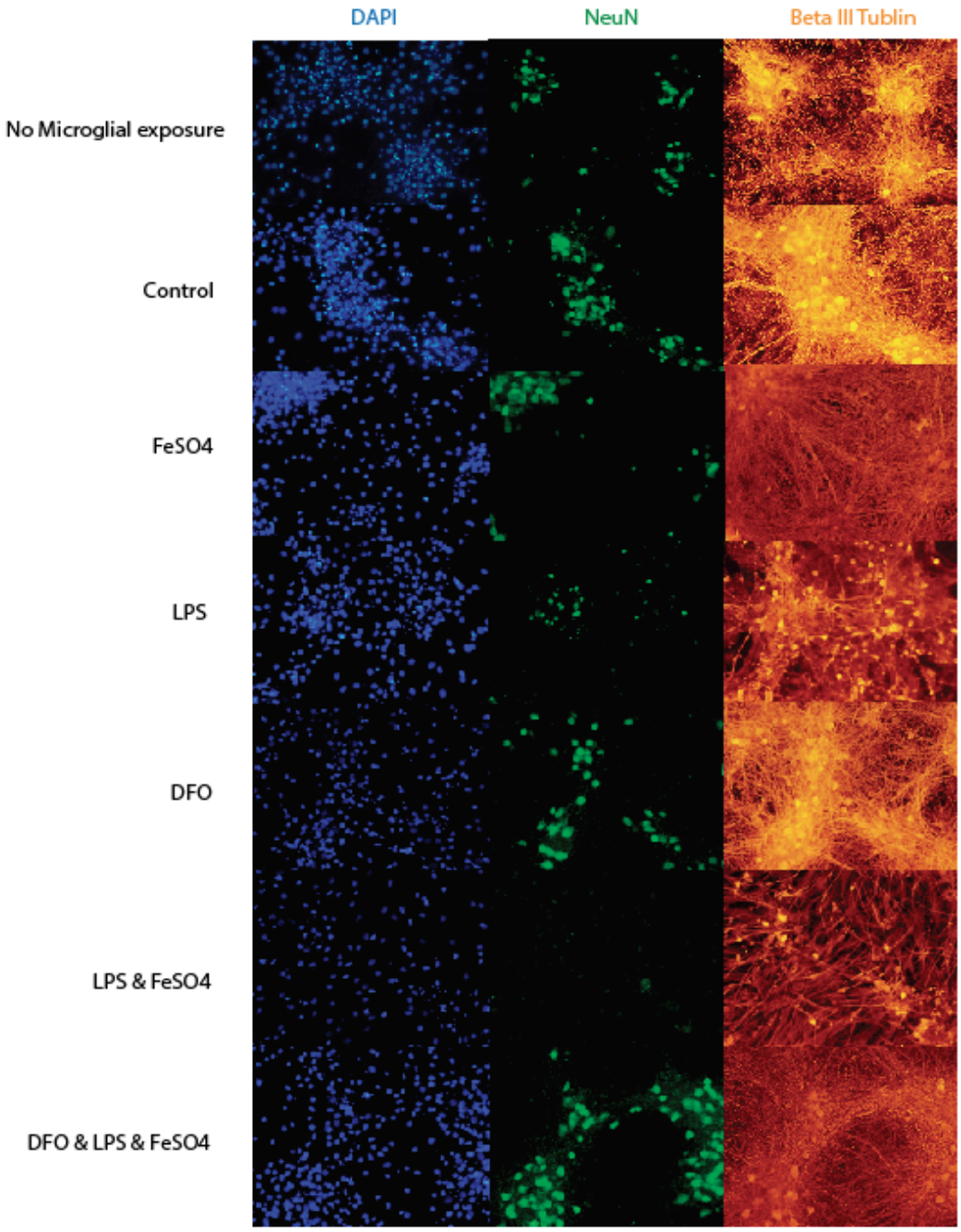

Figure 8. Iron alone and with simultaneous LPS treatment induces increased neuronal toxicty. Immunocytochemistry of co-cultured neurons presented. BV2's were treated in transwells with either FeSO4, LPS, or DFO alone on in combination. Blue, greeen, and red represent DAPI, NeuN, and beta III tublin respectively. FeSO4 and LPS alone caused an overall reduction in neurons. When treated simulataneously fewer neurons were present. Representative image of one trial.

Aim 2. Investigate the effect of NOX2 and NOX4 inhibition in microglia following iron exposure *in vitro* & Aim 3. Determine neuronal viability after exposure to media from microglia treated with iron, (in vitro).

We demonstrate that NOX2 and NOX4 proteins are present within BV2 immortalized microglia. While NOX2 proteins were significantly elevated at 24 hours following LPS exposure, NOX4 expression was unchanged between the groups. ROS production among the microglia illustrated an iron accentuated effect that was significantly ameliorated by the inhibition of NOX2 with GSK2795039 and NOX4 with GKT137831. This reduction in ROS suggested that NOX2 and NOX4 may be important within the mechanism of iron accentuated ROS production and their inhibition seems to be effective in reducing neuronal demise.

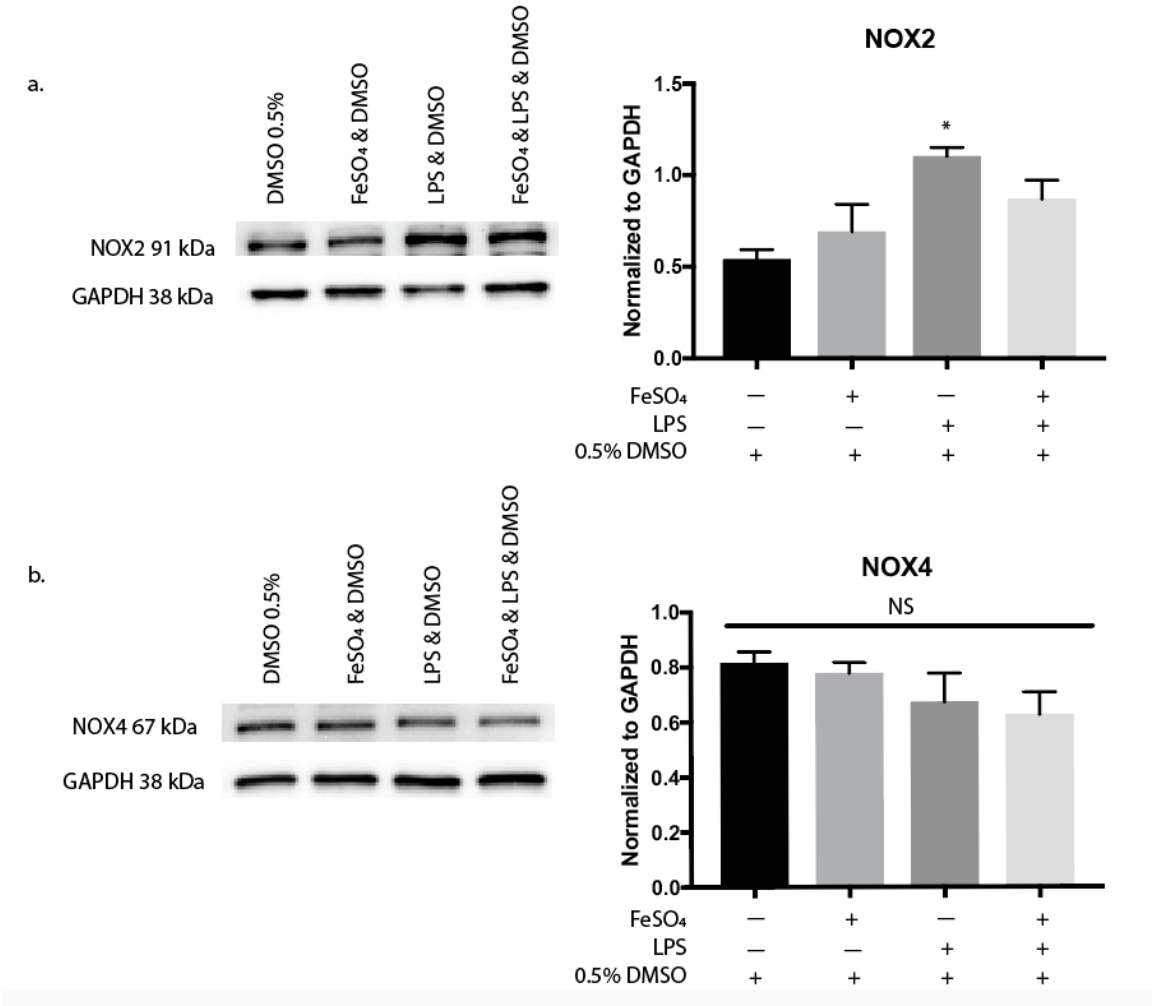

Figure 1: BV2 microglia express both NOX2 and NOX4 proteins. a) Western blotting of NOX2 revealed a significant increase in NOX2 protein after LPS exposure among BV2s. However, no differences were noted within the $FeSO_4$ & LPS & DMSO group. b) NOX4 expression among microglia treated with either $FeSO_4$, LPS, or DMSO alone or in combination revealed no significant (NS) differences between groups when normalized to GAPDH. All groups were compared using one-way ANOVA with Tukey's post-hoc test. Western blotting images each represent one trial, while the NOX2 bar graph represent an n=3 and the NOX4 graph represents an n=4. DMSO vs. *$p<0.05$. Bars represent mean +/- SEM.

Previous work illustrated the dynamic expression of NOX2 and NOX4 after TBI. (Cooney, Bermudez-Sabogal, & Byrnes, 2013) Although both are expressed after injury, there is a temporal component to be considered. NOX2 is known to be increased acutely following TBI; this is likely related to the initial mobilization of microglia to the injury site and subsequent

activation and assembly of NOX2. Although we detected an increase of NOX2 by LPS alone, this significant increase was not present within the $FeSO_4$ & LPS group. This suggested that increases in NOX2 protein concentrations were not a contributing factor to iron accentuated ROS production in microglia. NOX4, on the other hand, is reported to be increased chronically at 28 days post injury, therefore a lack of increased protein expression acutely at 24 hours *in vitro* is consistent with previous reports.(Cooney et al., 2013) NOX4 protein expression is also, therefore, not a contributing factor in elevations in ROS observed at 24 hours. NOX2 and NOX4 activity were not directly assessed in this study; future research will investigate the activity of the enzymes in these conditions.

LPS is a well characterized bacterial endotoxin known to activate toll-like receptor 4 and cause microglia to synthesize ROS. (Qin et al., 2004; T. Wang et al., 2004) Interestingly, we did not detect an increased level of ROS production within the LPS group, which could be due to the reported anti-inflammatory effect of 1% DMSO vehicle for GSK2795039 (Fig. 2a), however the combination of $FeSO_4$ & LPS & DMSO resulted in a significant increase in ROS synthesis despite the DMSO (Fig. 2a). (Elisia et al., 2016) This iron accentuated ROS production among microglia exposed to $FeSO_4$ & LPS & DMSO was significantly reduced following treatment with GSK2795039, suggesting that the presence of superoxide is essential for the mechanism in which iron can accentuate ROS production (Fig. 2a). NOX4 inhibition was equally effective in reducing iron accentuated ROS production, suggesting that hydrogen peroxide produced by NOX4 contributes to the mechanism of iron accentuated ROS production as well (Fig. 2b). Overall, the effective reduction of ROS after GSK2795039 and GKT137831 supports our hypothesis that iron undergoes the Fenton, Haber-Weiss reaction utilizing NOX2 and NOX4 derived superoxides and hydrogen peroxide to accentuate ROS production through a positive feedback with NOX proteins and perpetuate the
reaction.

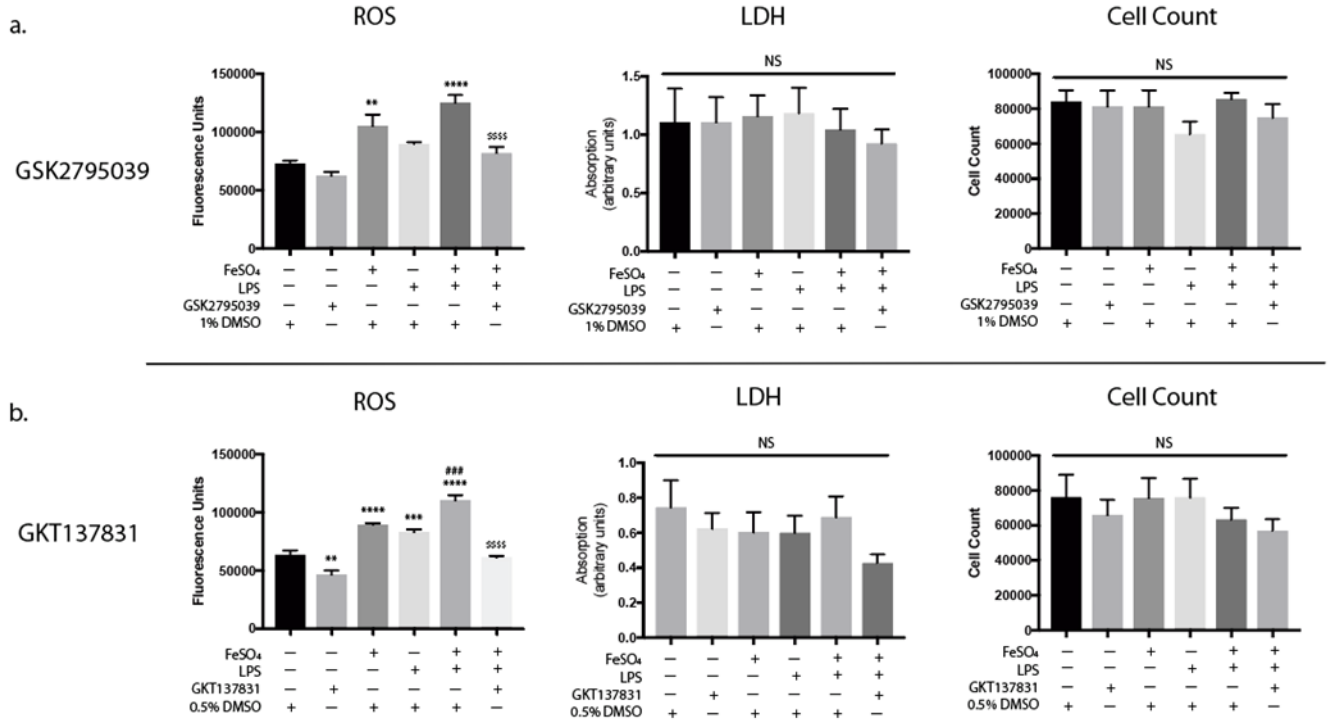

Figure 2. Iron accentuated ROS generation is significantly attenuated by NOX2 and NOX4 inhibition among activated microglia. a) $FeSO_4$ treatment among microglia induced a significant increase in ROS. LPS & DMSO did not increase ROS levels alone, however the addition of $FeSO_4$ to the group significantly increased ROS production. NOX2 inhibition with GSK2795039 significantly reduced ROS production to baseline. No significant differences in LDH or cell counts were detected between treatment groups within the GSK2795039 experiments. b) Robust ROS production among microglia treated with $FeSO_4$, LPS alone or in combination. The addition of NOX4 inhibition of GKT137831 significantly reduced the presence of ROS. Again, no significant differences in LDH or cell counts were detected between treatment groups within the GKT137831 experiments. In all graphs, symbols representing significance were assigned according to comparisons: DMSO group (*); $FeSO_4$ & DMSO (#); and $FeSO_4$ & LPS & DMSO ($). **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, $^{\#\#\#}p<0.001$ and $^{\$\$\$\$}p<0.0001$. All groups were compared using one-way ANOVA with Tukey's post-hoc test. All graphs represent n=6. Bars represent mean +/- SEM.

It is also important to note that GKT137831 has been described as dual NOX1 and NOX4 inhibitor. (Deliyanti & Wilkinson-Berka, 2015) However, NOX1 gene expression was not detected in quantitative polymerase chain reaction among BV2 microglia (data not shown), which suggests that NOX1 inhibition is not likely the result of GKT137831 administration in this immortalized cell line.

Increased oxidative stress and modifications to neuronal proteins have been reported as early as 3 hours after injury *in vivo*, with a peak at 24-48 hours. (Ansari, Roberts, & Scheff, 2008) Initially the depletion of antioxidants and ROS release from damaged mitochondria contribute to the early onset of oxidative stress, however we suspect iron accentuated ROS production plays an important role at 24 hours. To evaluate that impact of NOX2 and NOX4 inhibition on microglial related neurotoxicity at 24 hours, we conducted co-culture experimentation between microglia and primary cortical neurons (Fig. 3&4). Both NOX2 and NOX4 inhibition in microglia previously exposed to $FeSO_4$ and LPS significantly increased the presence of neurons in co-culture. This is consistent with recent findings that NOX2 and NOX4 mRNA silencing improved neuronal survivability by preventing apoptosis in a subarachnoid hemorrhage model. (G. Zhang et al., 2016) In addition, multiple studies suggest improvement of outcomes with various NOX2, NOX4 and non-specific NOX inhibitors, which may be related to their contributions to reduced iron accentuated ROS production after TBI *in vivo*.(Q. Wang et al., 2014; Q. G. Zhang et al., 2012) In addition to neuronal survivability, overall all cells within the mixed primary rat neuronal culture increased in number after the microglia were treated with either GSK2795039 or GKT137831. This suggests that activated microglia exposed to iron is universally cytotoxic to all cell types within the CNS and that reducing NOX activity may be

universally cytoprotective.

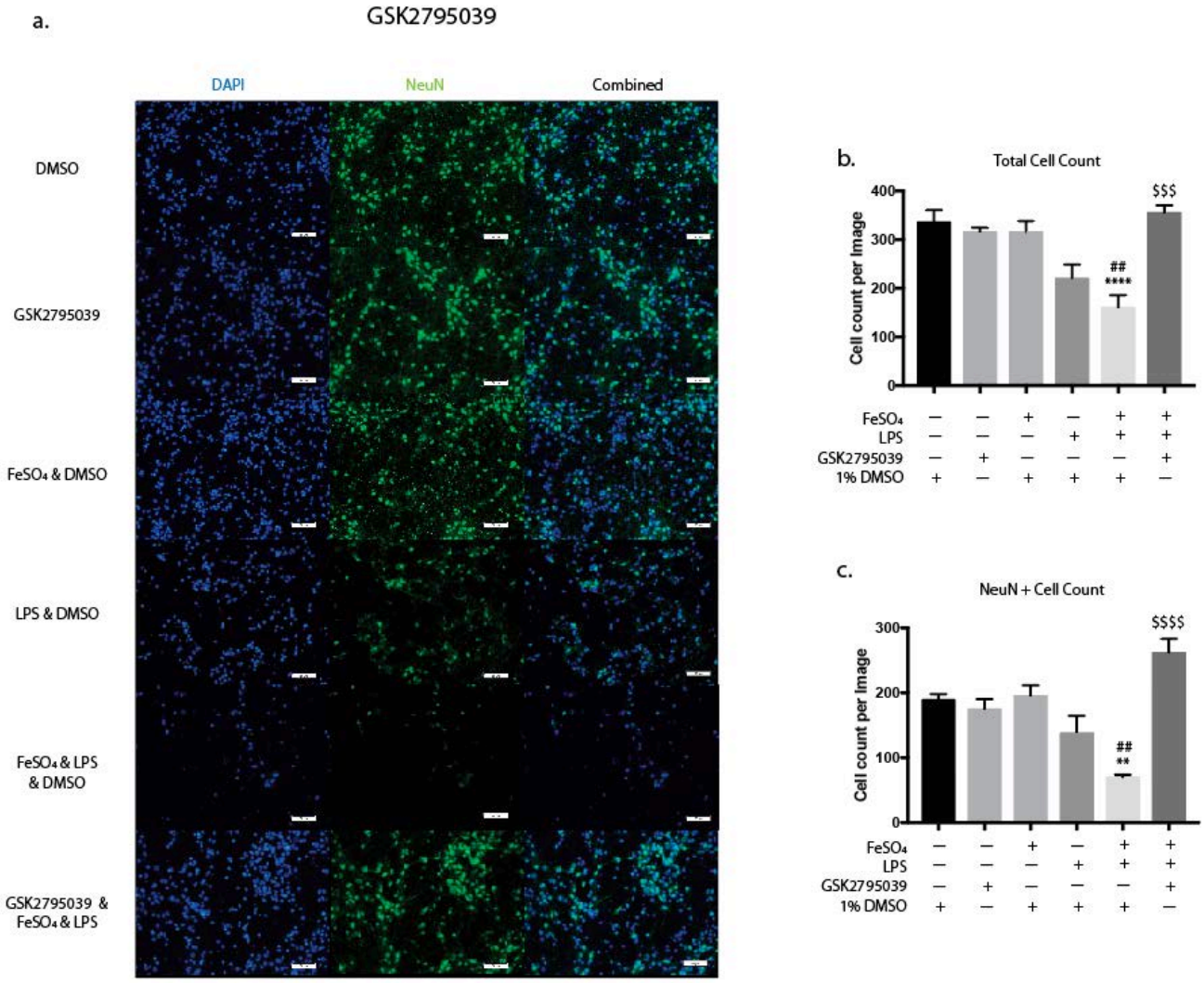

Figure 3. GSK2795039 improves neuronal survivability in co-culture with activated microglia. Primary neurons were co-cultured with microglia exposed to FeSO4, DMSO, LPS, GSK2795039, alone or in a combination. **a)** ICC of neurons after 24 hour co-culture. All groups were stained with DAPI and NeuN. Qualitatively, a generalized reduction in the expression of NeuN was noted on those groups co-cultured with microglia treated with FeSO4, LPS, or in combination. The addition of GSK2795039 significantly improved neuronal survivability. **b)** Improved survivability among all cell types present in the neuronal culture were observed by the increased cell counts in groups treated with GSK2795039. **c**) Quantitative analysis of NeuN positive cell counts revealed a significant reduction in neuronal cell count after co-culture with microglia treated with $FeSO_4$ & LPS & DMSO. GSK2795039 prevented neuronal loss after co-culture and significantly improved neuronal cell counts. All ICC images are representative of one trial. All groups were compared using one-way ANOVA with Tukey's post-hoc test. In all graphs, symbols representing significance were assigned according to comparisons: DMSO group (*); $FeSO_4$ & DMSO (#); and $FeSO_4$ & LPS & DMSO ($). **$p<0.01$, ****$p<0.0001$, $^{\#\#}p<0.01$, $^{\$\$\$}p<0.001$ and $^{\$\$\$\$}p<0.0001$. All graphs represent n=3. Bars represent mean +/- SEM. Size bars represent 50 µm.

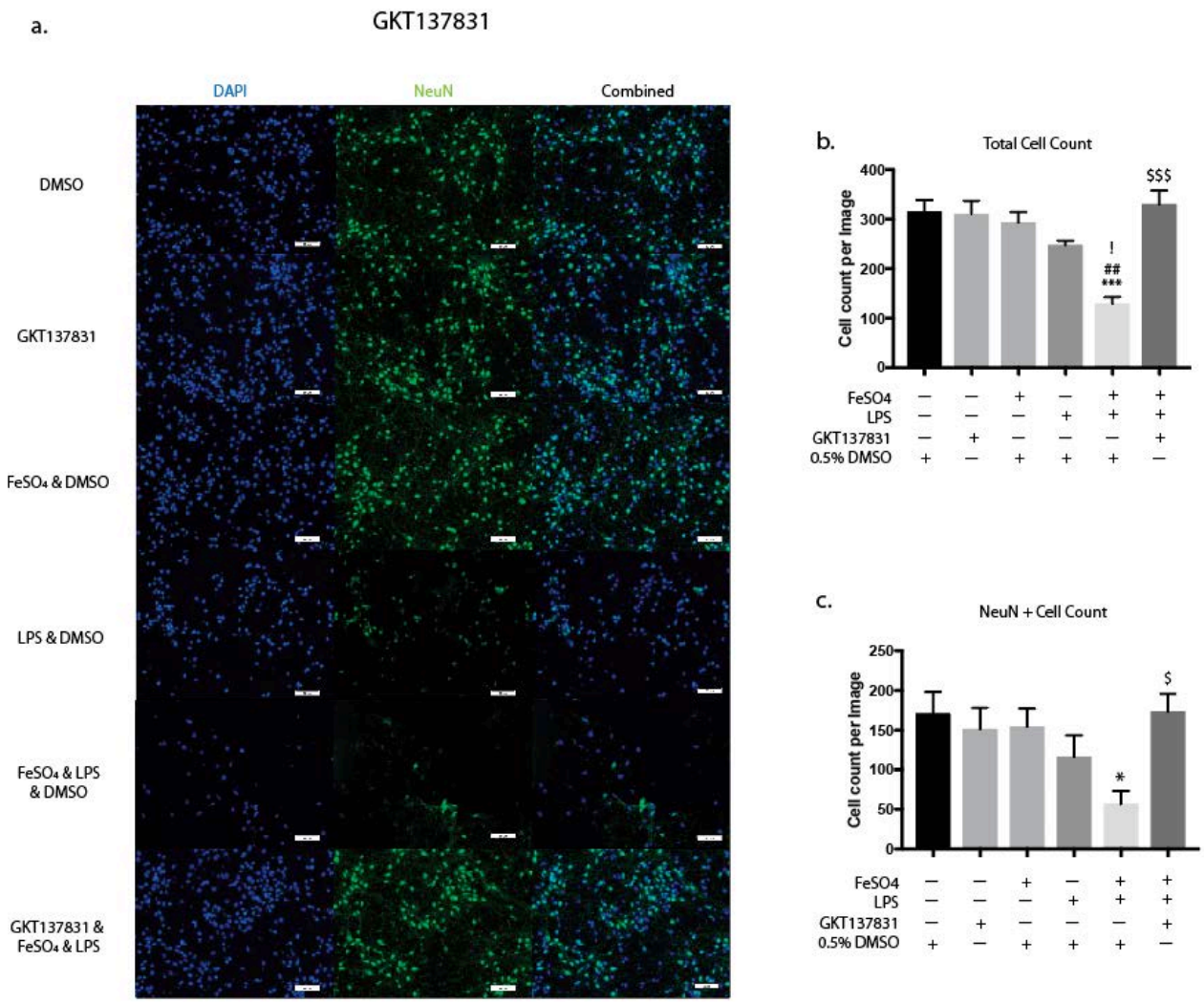

Figure 4. GKT137831 improves neuronal survivability in co-culture with activated microglia. Primary neurons were co-cultured with microglia exposed to FeSO4, DMSO, LPS, GKT, or in combination. **a)** ICC of neurons after 24 hour co-culture. All groups were stained with DAPI and NeuN. Qualitatively, a generalized reduction in the expression of NeuN was noted on those groups co-cultured with microglia treated with $FeSO_4$ & LPS & DMSO. **b)** Improved survivability among all cell types present in the neuronal culture were observed by the increased cell counts in groups treated with GKT137831. **c**) Quantitative analysis of NeuN counts revealed a significant reduction in the presence of neurons after co-culture with microglia treated with $FeSO_4$ & LPS & DMSO. GKT137831 reduced neuronal toxicity and prevented neuronal loss after co-culture. All ICC images are representative of one trial. All groups were compared using one-way ANOVA with Tukey post-test. In all graphs, symbols representing significance were assigned according to comparisons: DMSO group (*); $FeSO_4$ & DMSO (#); LPS & DMSO (!), and $FeSO_4$ & LPS & DMSO ($). *$p<0.05$, ***$p<0.001$, $^{\#\#}p<0.01$, $^{!}p<0.05$, $^{\$}p<0.05$ and $^{\$\$\$}p<0.001$. All graphs represent n=3. Bars represent mean +/- SEM. Size bars represent 50 µm.

Due to the heterogeneous nature of primary mixed neuronal cell cultures, we co-cultured microglia with differentiated PC12 neuronal like cells to isolate the effect of iron accentuated ROS production on neurons. PC12s are neuron-like cells capable of neurite extension and therefore an ideal model to measure the severity of oxidative stress through the apoptotic marker cleaved caspase 3.(Piga, Saito, Yoshida, & Niki, 2007) Despite PC12's reportedly robust nature, the cultures displayed a significantly increased count of cleaved caspase 3 positive cells after co-culture with microglia previously treated with $FeSO_4$ & LPS & DMSO (Fig. 5 & 6). Inhibition

of either NOX2 with GSK2795039 or NOX4 with GKT137831 in microglia prior to co-culture reduced the cellular toxicity among PC12s as evidenced by fewer apoptotic cells. This illustrated the cytotoxic potency of the ROS accentuation by $FeSO_4$ & LPS and its indirect effect of a cell line reported to have unusually effect antioxidant capabilities.

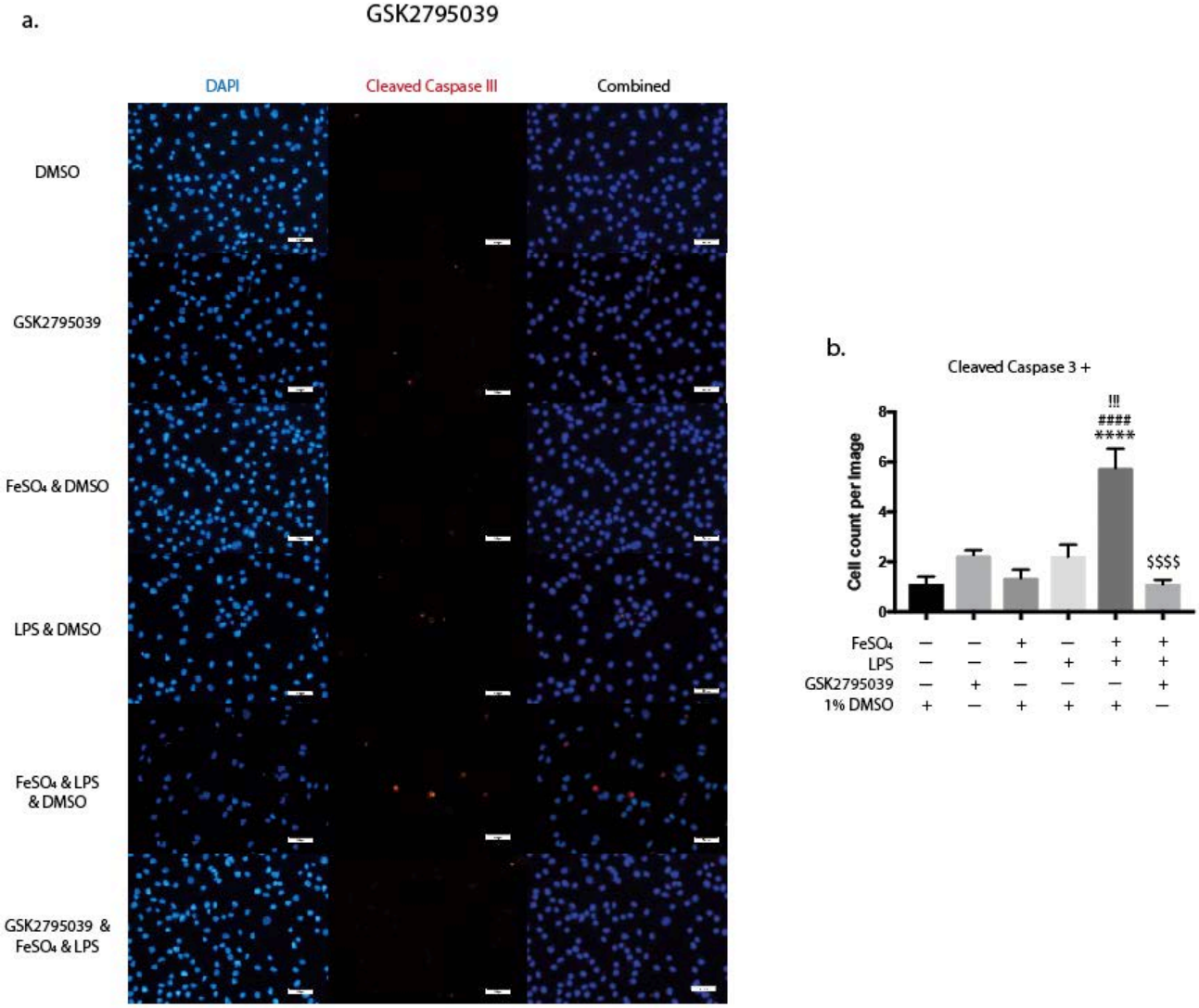

Figure 5. GSK2795039 reduces apoptosis among differentiated PC12 cells after activated microglia co-culture. PC12 cells were differentiated to a neuronal like phenotype for one week prior to microglia co-culture. **a**) ICC revealed increased staining for cleaved caspase-3 among PC12 co-cultured with microglia treated with $FeSO_4$, LPS, or in combination. The addition of GSK2795039 to the microglia treatment groups reduced the presence of cells positive for cleaved caspase-3. **b**) Quantitative analysis of PC12 cells positive with cleaved caspase-3 were significantly increased when co-cultured with microglia treated with $FeSO_4$ & LPS & DMSO. However, this increased apoptosis among PC12 cells was reduced by the addition of GSK2795039. All immunocytochemistry images are representative of one trial. All groups were compared using one-way ANOVA with Tukey's post-hoc test. In all graphs, symbols representing significance were assigned according to comparisons: DMSO group (*); $FeSO_4$ & DMSO (#); LPS & DMSO (!), and $FeSO_4$ & LPS & DMSO ($). ****$p<0.0001$, $^{\#\#\#\#}p<0.0001$, $^{!!!}p<0.001$, and $^{\$\$\$\$}p<0.0001$. All graphs represent n=4. Bars represent mean +/- SEM. Size bars represent 50 µm.

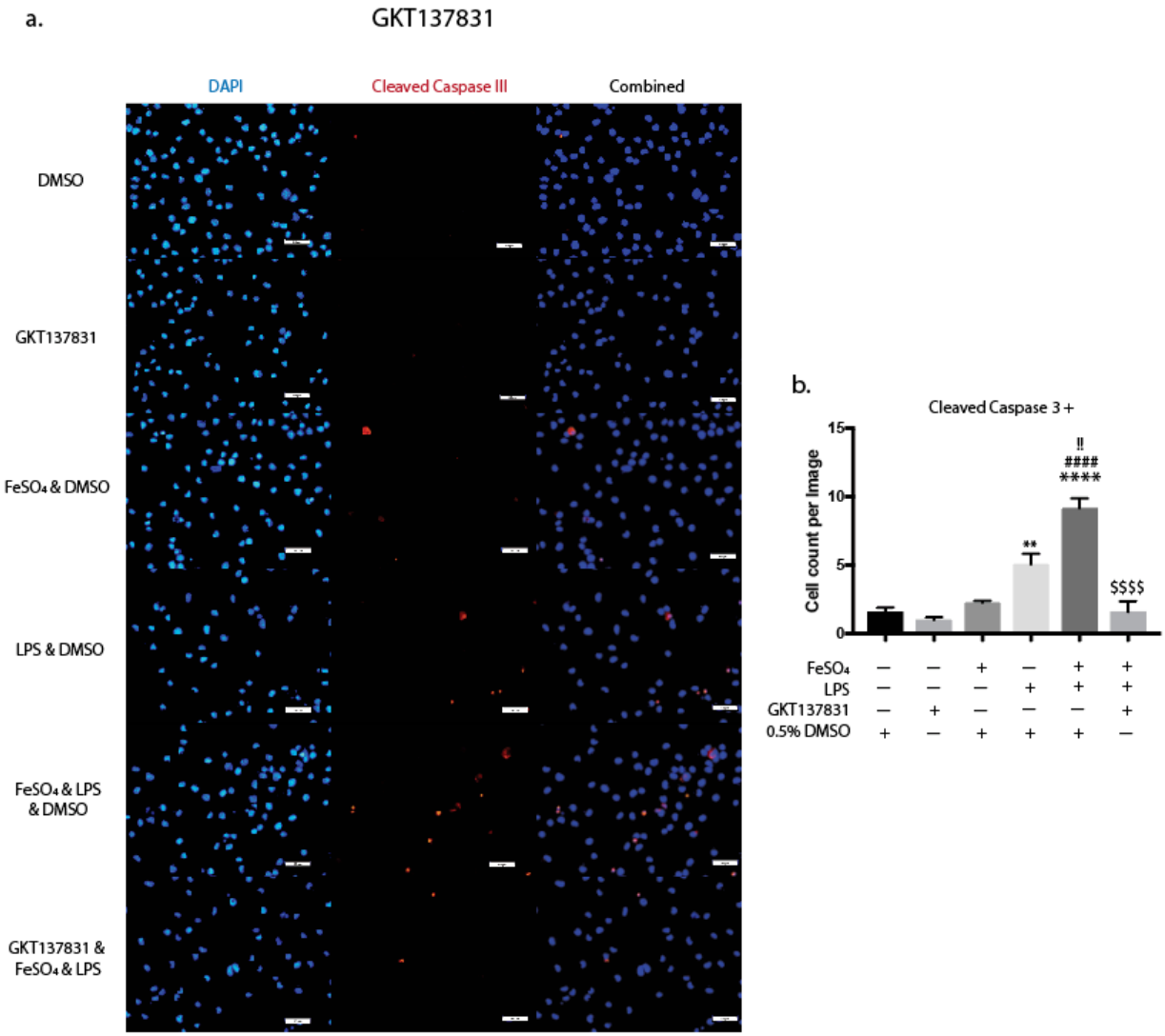

Figure 6. GKT137831 reduces apoptosis among differentiated PC12 cells after activated microglia co-culture. PC12 cells were differentiated to a neuronal like phenotype for one week prior to microglia co-culture. **a.** ICC revealed increased staining among PC12 co-cultured with microglia treated with LPS, or in combination with $FeSO_4$. The addition of GKT137831 to the microglia treatment groups reduced the presence of cells positive for cleaved caspase-3. **b**. Quantitative analysis of PC12 cells positive with cleaved caspase-3 were significantly increased when co-cultured with microglia treated with $FeSO_4$ & LPS & DMSO. However, this increased apoptosis among PC12 cells was reversed by the addition of GKT137831. All ICC images are representative of one trial. All groups were compared using one-way ANOVA with Tukey post-test. In all graphs, symbols representing significance were assigned according to comparisons: DMSO group (*); $FeSO_4$ & DMSO (#); LPS & DMSO (!), and $FeSO_4$ & LPS & DMSO ($). **$p<0.01$, ****$p<0.0001$, $^{\#\#\#\#}p<0.0001$, $^{!!}p<0.01$, and $^{\$\$\$\$}p<0.0001$. All graphs represent n=3. Bars represent mean +/- SEM. Size bars represent 50 µm.

The data presented here provides evidence that microglia, and possibly all cells expressing NOX2 or NOX4, may passively and indirectly exacerbate neuronal toxicity within a TBI focal lesion and surrounding penumbra. Iron has been identified as an important contributor to oxidative stress in neurodegenerative diseases and seems to be a significant player within TBI pathophysiology as well. Its focus as a primary contributor to oxidative stress is not novel,

however its mitigation in TBI may yield improved therapeutic outcomes by utilizing NOX2 or NOX4 inhibitors.

Effect of problems or obstacles on the results:

Despite the generation of solid data during the first year, we did encounter difficulties during this period of time. Initially, we experienced inconsistent data from all cellular outcome measures after plating and exposing cells $FeSO_4$. After a thorough review of protocols and techniques with Dr. Byrnes, we identified that the BV2 cells were over confluent before cellular outcome experimentation and therefore a reduction in plating density was necessary. After reducing the plating density, our data became more consistent and revealed that iron alone was capable of inducing ROS production. Another issue encountered was the ineffectiveness of the MTS viability assay to detect cellular activity. To ameliorate this problem we purchased a cell counter and utilized methylene blue staining to determine cellular viability indirectly.

In the final year we overcame multiple obstacles. We identified inconsistent results from the NOX2 inhibitor gp91 dstat. After consultation, the manufacturer confirmed that several lots of gp91 dstat were ineffective. This drug was replaced with newer lots but continued to have inconsistent results. Unfortunately, the issues with gp91 dstat could not be overcome within a reasonable timeframe, therefore we identified a well-established NOX2 inhibitor GSK2795039 as a replacement drug, which produced results consistent with previously reported data. In addition to the pharmacologic difficulties, the neuronal cultures were difficult to create. The primary cultures intermittently suffered from severe cellular necrosis approximately 7 days post-harvest, despite multiple alterations to the protocol to ameliorate cellular demise. After considering multiple possibilities, we were unable to identify the underlying cause of the neuronal necrosis. Fortunately, we received primary neuron cultures from Dr. Bogdan Stocia from University of Maryland School of Medicine Anesthesia research department to complete the experimentation. The cells were transported via portable incubator and delivered to USUHS. After a 24-hour stabilization period, the cells were exposed to treatments according to protocol time points.

Conclusion:

In conclusion, iron is important for cellular homeostasis and necessary for life. Despite its salubrious effects, iron is extremely reactive and is known to produce substantial oxidative stress and damage in many neurodegenerative and injury models. Fenton Haber-Weiss chemistry is responsible for significant production of ROS when provided ideal conditions, which include NOX derived ROS and non-protein bound iron. These conditions are present in TBI and contribute to oxidative stress mediated neurotoxicity. Iron amplified ROS production is a dose dependent phenomenon that appears to not exacerbate microglial M1 polarization, however it may prolong the pro-inflammatory response chronically and prevent M2 microglial polarization. The pro-inflammatory microenvironment of the lesion may be perpetuated by increased oxidative stress. NOX2 and NOX4 play an important role with iron pathophysiology in TBI. NOX2 superoxides and NOX4 hydrogen peroxides directly contribute to iron amplification of ROS production. Inhibition of either NOX isoforms results in significant reduction of ROS production among microglia and subsequently reduced toxicity among primary neurons and hardy PC12 neuron like cells. DFO, GSK2795039, and GKT137831 all have potential as therapeutic options for TBI. Currently, DFO is under phase 2 human clinical trials to determine the feasibility and efficacy of treatment following hemorrhagic shock. GSK2795039 and

GKT137831 offer promising pharmacologic profiles and are likely candidates as a treatment in the near future. Any of these options would greatly improve the dearth of treatment options available to the millions of patients living with TBI.

Significance of Study or Project Results to Military Nursing

Hemorrhage remains the leading cause of death among combat casualties. Administration of lifesaving PRBCs by nurses and CRNAs among poly-trauma causalities occurs frequently in the deployed setting. Replacement of hemorrhaged blood with PRBCs can increase the percentage of circulating free iron leading to toxicity. PRBC administration will likely remain a mainstay of resuscitation until a suitable alternative is invented. Therefore, it is imperative that the deleterious effects of free iron are elucidated in order to increase medical readiness through evidence-based practice and ultimately improve patient outcomes. This project establishes a foundation for future research and may significantly affect nursing practice through modification of blood product administration. Our novel research suggested a potential therapeutic advantage by free iron scavenging therapy, antioxidant administration, or NOX inhibition in the presence of excessive free iron.

Changes in Clinical Practice, Leadership, Management, Education, Policy, and/or Military Doctrine that Resulted from Study or Project

No immediate changes in clinical practice, management, or policy resulted from this research due to its nascent nature. However, the research does offer insight into the importance of ROS mitigation among TBI patients to improve neuronal survivability and improve long-term outcomes following injury. In addition, interesting questions concerning future research topics were generated because of this research, such as whether daily oral antioxidant supplementation for deployed soldiers can reduce cerebral oxidative stress following injury.

Summary of Dissemination

Type of Dissemination	**Citation**	**Date and Source of Approval for Public Release**
Publications	von Leden, R.E., et al., *Central Nervous System Injury and Nicotinamide Adenine Dinucleotide Phosphate Oxidase: Oxidative Stress and Therapeutic Targets.* J Neurotrauma, 2016.	March 2016, USUHS External Affairs
Podium Presentations	Young Yauger, Investigating the Effects of Iron on Microglia in vitro TSNRP Research and Evidence Based Practice Dissemination Course, San Antonio Texas, August 2016	July 2016, USUHS External Affairs
	Young Yauger, Inhibition of NOX4 reduces iron accentuated oxidative stress among neurons *in vitro* TSNRP Research and Evidence Based Practice Dissemination Course, Ellicott City MD, April 2017	March 2017, USUHS External Affairs

Poster Presentations	Young Yauger, Sara Bermudez, Kimberly Byrnes. NADPH Oxidase 4 inhibition reverses iron induced perturbations of reactive oxygen species within activated microglia. National Neurotrauma Society Annual Conference. Santa Fe, NM, August 2015.	July 2015, USUHS External Affairs
	Young Yauger, Sara Bermudez, Kasey Moritz, Kimberly Byrnes. Iron accentuates reactive oxygen species generation without inducing a polarization shift within microglia. National Neurotrauma Society Annual Conference. Lexington, KY, July 2016	July 2016, USUHS External Affairs
	Young Yauger, Alexandra Yaszemski, Sara Bermudez, Kimberly Byrnes. Inhibition of NOX4 reduces oxidative stress and neuronal damage induced by iron-activated microglia. National Neurotrauma Society Annual Conference. Snowbird, UT, July 2017	July 2017, USUHS External Affairs

Reportable Outcomes

Reportable Outcome	Detailed Description
Applied for Patent	None
Issued a Patent	None
Developed a cell line	None
Developed a tissue or serum repository	None
Developed a data registry	None

Recruitment and Retention Table

Recruitment and Retention Aspect	Number of Subjects This Reporting Period	Total Number of Subjects Since Study or Project Began
Animals Projected in Grant Application	8	8
Animals Purchased	8	8
Model Development Animals	0	0

Summary regarding recruitment and retention: After IACUC approval was attained on 26 September 2016, we purchased 8 pregnant Sprague Dawley rats to create primary microglia cultures for our study.

Final Budget Report

HENRY M. JACKSON FOUNDATION FOR THE ADVANCEMENT OF MILITARY MEDICINE
Advancing Military Medical Research

TASK BUDGET SUMMARY

Current as of: OCT2017

Organization: HJF-Henry M. Jackson Foundation
Award #/Name: 64761 - IRON ACTIVATION
Award Manager: YAUGER, YOUNG J
Award Period: 08/15/2015 to 08/14/2017
Project #/Name: 308009 - IRON ACTIVATION OF CELLULAR
Project Manager: YAUGER, YOUNG J
Project Period: 08/15/2015 to 08/14/2017

Task # / Name: 1.00 - IRON ACTIVATION
Task Period: 08/15/2015 to 08/14/2017
Task Manager: YAUGER, YOUNG J
Task Desc: APG-7-3568 (N15-P14)
Award Sponser: UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
Billing Analyst: ARCIDIACONO, DONNA M.
Primary Analyst: SOLISZEWSKA-JOHNSON, LIDIA M.
Ref Award#: HU0001-15-1-TS16

Current As of	Award/Project/Task Number	Task Budgetary Control	Category Group	Expenditure Category	Budgetary Control	Current Month Expenses	Budget	Open Commitment	Task-To-Date Expenses	Total Funds Used	Balance Available	Percentage Available
10/17/2017	64761 - 308009 - 1.00	Absolute	DIRECT	SUPPLIES	Advisory	**0.00**	29,871.76	0.00	29,871.14	29,871.14	0.62	0.00
				OTHER DIRECT COSTS	Absolute	**0.00**	132.24	0.00	132.24	132.24	0.00	0.00
				TOTAL DIRECT :		**0.00**	**30,004.00**	**0.00**	**30,003.38**	**30,003.38**	**0.62**	**0.00**
			INDIRECT	ON-SITE OVERHEAD	Advisory	**0.00**	10,940.00	0.00	10,050.61	10,050.61	889.39	8.13
				COMPANY-WIDE G & A	Advisory	**0.00**	5,854.00	0.00	5,622.66	5,622.66	231.34	3.95
				TOTAL INDIRECT :		**0.00**	**16,794.00**	**0.00**	**15,673.27**	**15,673.27**	**1,120.73**	**6.67**
				TOTAL TASK :		**0.00**	**46,798.00**	**0.00**	**45,676.65**	**45,676.65**	**1,121.35**	**2.40**

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