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14. ABSTRACT

Studies suggest that neuroinflammation-induced brain injury could be an initiating factor in developing Alzheimer's disease-related pathologies and provides substantial rationale to identify and characterize post-injury inflammatory biomarkers associated with repetitive brain trauma. To date, mechanisms linking TBI to AD remain unclear. Professional fighters experience repetitive brain injury that results in altered brain structures. We hypothesized that TBI-induced inflammatory response is critical in mediating AD-related pathology and specific inflammatory proteins can be used as post-injury biomarkers. The goal of the current study was to characterize inflammatory states in peripheral blood monocytes and plasma of active and retired MMA fighters and boxers from the Professional Fighters Brain Health Study. We examine 40 immune/inflammation-related biomarkers in the plasma of fighters and controls. We found changes specific to active fighters but some that persisted in retired fighters indicating some alterations persist chronically. Likewise, peripheral blood monocytes showed impaired TREM2 expression in both active and retired fighters, indicating that the deficiency is chronic.

15. SUBJECT TERMS
None Listed

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1. INTRODUCTION

Little is currently known regarding the role of inflammation in the progression of traumatic brain injury induced neurodegenerative disorders. Alzheimer's Disease (AD) is a common outcome of patients who have experienced mild to moderate brain injury, as well as those who have suffered from repeated concussive injury. There is a persuasive body of evidence favoring a significant inflammatory component in AD. A large number of inflammatory cells including microglia, astrocytes, and infiltrating peripheral immune cells as well as inflammatory molecules are present at elevated levels in the AD brain, highlighting the potential role of neuroinflammation in regulating AD pathologies as a result of brain trauma. This role of neuroinflammation is supported by several observations. First, a recent set of experiments demonstrated that tau (MAPT) pathology temporally co-exists with gliosis following mild repetitive TBI in the human tau (hTau) mouse model of MAPT pathology and AD. Our studies supported by a previous DoD grant (W81XWH-14-1-0265) have confirmed that even a single TBI enhances accumulation of activated macrophages and phosphorylated MAPT in the hTau mouse model. Similar findings have been reported in wild-type mice after blast induced brain injury, as well as in a triple transgenic mouse model of AD following a single moderate TBI. Second, numerous reports have demonstrated activated microglia located near the injury release several proinflammatory cytokines and chemokines. These inflammatory components in turn can exacerbate MAPT pathologies. Third, post-injury neuronal accumulation of amyloid beta (Aβ) correlates with increased numbers of microglia expressing the cytokine IL-1α. Finally, our studies (DoD grant W81XWH-14-1-0265) show that a single moderate TBI induces expression of key pro-inflammatory cytokines at acute (3 days post-injury, DPI) time points in non-transgenic mice. Together, these studies suggest that neuroinflammation induced with brain injury could be an initiating factor in AD-related pathologies and provides substantial rationale for studies aiming to identify and characterize post-injury inflammatory biomarkers associated with repetitive brain trauma and AD. The goal of the current study was to characterize inflammatory states in peripheral blood monocytes and plasma of active and retired MMA fighters and boxers. Our observations add critical knowledge to the fields of neuroinflammation and traumatic brain injury as well as identify potential biomarkers of disease and novel therapeutic targets.

2. KEYWORDS

Traumatic brain injury
Alzheimer's disease
Inflammation
Microglia
Monocytes
Neurodegeneration
TREM2
immunity

3. ACCOMPLISHMENTS

What were the major goals of the project?

The overall goal of the project was to identify unique inflammatory biomarkers that correlate with the development of AD-like pathology after repetitive head injury. The project capitalizes on the Professional Fighters Brain Health Study (PFBHS) based out of the Luo Ruvo Center for Brain Health at the Cleveland Clinic in Las Vegas, Nevada, which is a longitudinal cohort study of boxers and mixed martial arts (MMA) fighters. The PFBHS has shown that both active and retired fighters show increased brain atrophy, supporting the hypothesis that repetitive head injury is a risk factor for the development of AD-like pathology. Inflammation after head injury is thought to be a contributing factor to increased risk for AD-like pathology. To identify inflammatory biomarkers in professional fighters, we will query expression of inflammation-linked protein TREM2 on monocyte populations and the concentration of myriad immune proteins in plasma of active and retired professional fighters to determine acute and chronic post-injury profiles, respectively.

<u>Major Goals of the Project</u>

1. Approvals: HRPO and IRB approval for all studies; month 1-6; 100% complete

2. Staff training: Optimized procedures for sample collection, transfer, and processing; month 3-12; 100% complete

3. Preliminary study to detect biomarkers: Inflammatory biomarkers confirmed in peripheral blood; month 12-15; 100% complete

4. Blood sample collection: Collect blood from separate groups of active or retired profession fighters and age-matched controls; month 15-33; 100% complete

5. Complete imaging studies: Complete magnetic resonance imaging (MRI) and positron emission tomography (PET) amyloid imaging in a subset of retired fighters; month 18-33; 50% complete

6. Collect and process cerebral spinal fluid (CSF): Collect and process CSF for expression of inflammatory biomarkers in a subset of retired fighters; month 18-33; 30% complete

7. Data analysis: Complete data analysis and begin manuscript preparation; month 30-36; 90% complete

8. Manuscript publication: Submit manuscript for publication; month 33-36; 50% complete

What was accomplished under these goals?

There were three institutions involved in this project: the Bruce Lamb lab at Indiana University (IU), Indianapolis, IN; the Jefferson Kinney lab at University of Nevada Las Vegas (UNLV), Las Vegas, Nevada; and the Charles Bernick group associated with the PFBHS at the Luo Ruvo Center for Brain Health (LRCBH) at the Cleveland Clinic in Las Vegas, Nevada. At the beginning of this project, the Lamb lab was located at the Cleveland Clinic in Cleveland, Ohio and moved to IU in January of 2016. This resulted in a significant delay in accomplishing the specific tasks of each goal. Eventually, HRPO and IRB approvals were obtained by each site separately over the course of the project period (Goal 1).

There were essentially three parts to this project: sample collection at LRCBH, blood cell analysis of TREM2 expression at UNLV, and biomarker analysis at IU. Over the time frame of this project, blood samples were collected from active and retired boxers and MMA fighters as well as controls. Originally, we had planned for the UNLV group to isolate leukocytes from the blood samples to ship to IU for analysis of TREM2. In 2017, we completed a series of pilot experiments to determine the feasibility and reproducibility of this process (Goal 3). While we demonstrated success with this practice, it was decided later in 2017 that it would be more practical to analyze the blood samples immediately after leukocyte isolation at UNLV. Dr. Shane Bemiller went to UNLV in 2017 and helped Dr. Kinney's lab set up the assays for analysis of TREM2 expression using flow cytometry (Goal 2). Dr. Bemiller also trained Dr. Kinney's group to perform the analysis as well (Goal 2).

At LRCBH, the blood samples were divided into whole blood aliquots or processed for plasma aliquots for various projects the PFBHS supports. For this project, the Lamb lab at Indiana University in Indianapolis received aliquots of frozen plasma. We received 269 plasma samples that were collected from 2013 until October 2019 (Goal 4). Starting in 2018, whole blood portions of 85 of these samples were picked up by the Kinney Lab at University of Nevada Las Vegas for TREM2 expression analysis (Goal 4).

Throughout the course of the project, we have been guided by Ms. Heather Rodney as our point of contact at the Federal Interagency Traumatic Brain Injury Research Informatics System (FITBIR) to upload our data to the system. We have created pseudoGUIDs for all of the subjects that did not have the unique identified assigned previously. We have mapped the FITBIR Demographics form structure for our study. We are currently set to upload our data in a few weeks. This will complete our requirements for the FITBIR submission.

The goals of the project included obtaining a high-resolution structural MRI and PET amyloid imaging of the brain at the LRCBH (Goal 5) and cerebrospinal fluid (CSF) (Goal 6) from a subset of the study participants. The MRI and PET scans were not supported through the funds allocated to this project as there were part of the parent PFBHS. Due to the extreme turnover and turmoil at LRCBH, we have not been able to obtain the data

for inclusion in this project at the time of this report. We also encountered issues with obtaining the CSF samples, while some samples were collected, there was reluctance on the study participants to donate this type of sample due to the invasive nature of lumbar puncture. Over the course of the project, we were only able to obtain approximate 30% of the CSF samples from fighters and 20% from controls. Since we aimed to collect n=8 fighters and n=7 controls, we do not have enough samples for meaningful analysis of AD-related biomarkers.

Table 1 shows the characteristics of the study group. Descriptive statistics were calculated for continuous outcomes such as age with mean and standard deviation (SD) and for dichotomized results such as sex with proportions. There were 5 groups: controls, retired boxers, active boxers, retired MMA fighters, and active MMA fighters.

Table 1. Characteristics of the study cohort.

	control	*boxer retired*	*boxer active*	*MMA retired*	*MMA active*
male	20 (71%)	52 (93%)	33 (85%)	19 (95%)	65 (77%)
female	8 (29%)	4 (7%)	6 (15%)	1 (5%)	19 (23%)
education	15.61 (2.45)	12.69 (2.47)	13.23 (2.24)	13.93 (1.95)	13.48 (2.36)
age, years	38.32 (15.93)	49.71 (8.22)	33.56 (5.80)	37.6 (6.99)	33.17 (4.87)
ethnicity					
African-American	1 (3%)	23 (41%)	13 (33%)	2 (10%)	11 (13%)
White	24 (86%)	32 (57%)	23 (59%)	16 (80%)	66 (80%)
Asian	3 (11%)	1 (2%)	1 (3%)	-	-
Other	-	-	2 (5%)	2 (10%)	6 (7%)
# fights*	0	40.56 (14.93)	20 (18.44)	21.29 (24.11)	17.65 (14.75)

To determine the levels of inflammatory-related molecules in the blood plasma of the fighter groups compared to healthy controls, biomarker analysis was performed using ELISA assays via the Meso Scale Diagnostics platform. Four general categories were queried including proinflammatory (IFNγ, IL1β, IL2, IL4, IL6, IL8, IL10, IL12p70, IL13, TNFα), cytokine (GM-CSF, IL1a, IL5, IL7, IL12/IL23p40, IL15, IL16, IL17a, TNFβ, VEGF-A), angiogenesis (FGF-basic, PIGF, Tie2, VEGF-A, VEGF-C, VEGF-D, VEGFR1/Flt1), chemokine (IL8, CCL2, CCL13, CCL22, MIP1b, CCL11, eotaxin-3, CCL17/TARC, CXCL10), and vascular injury (CRP, ICAM1, SAA, VCAM1). Measures across the five study groups were compared using a multiple comparisons one-way ANOVA for each analyte with group as the primary variable of interest. Tukey-Kramer post-hoc tests were used to adjust the multiple comparisons for the *P* value and the confidence interval (95%). Additional analyses will be computed using multiple linear regression model to control for other covariates such as age, race, and education.

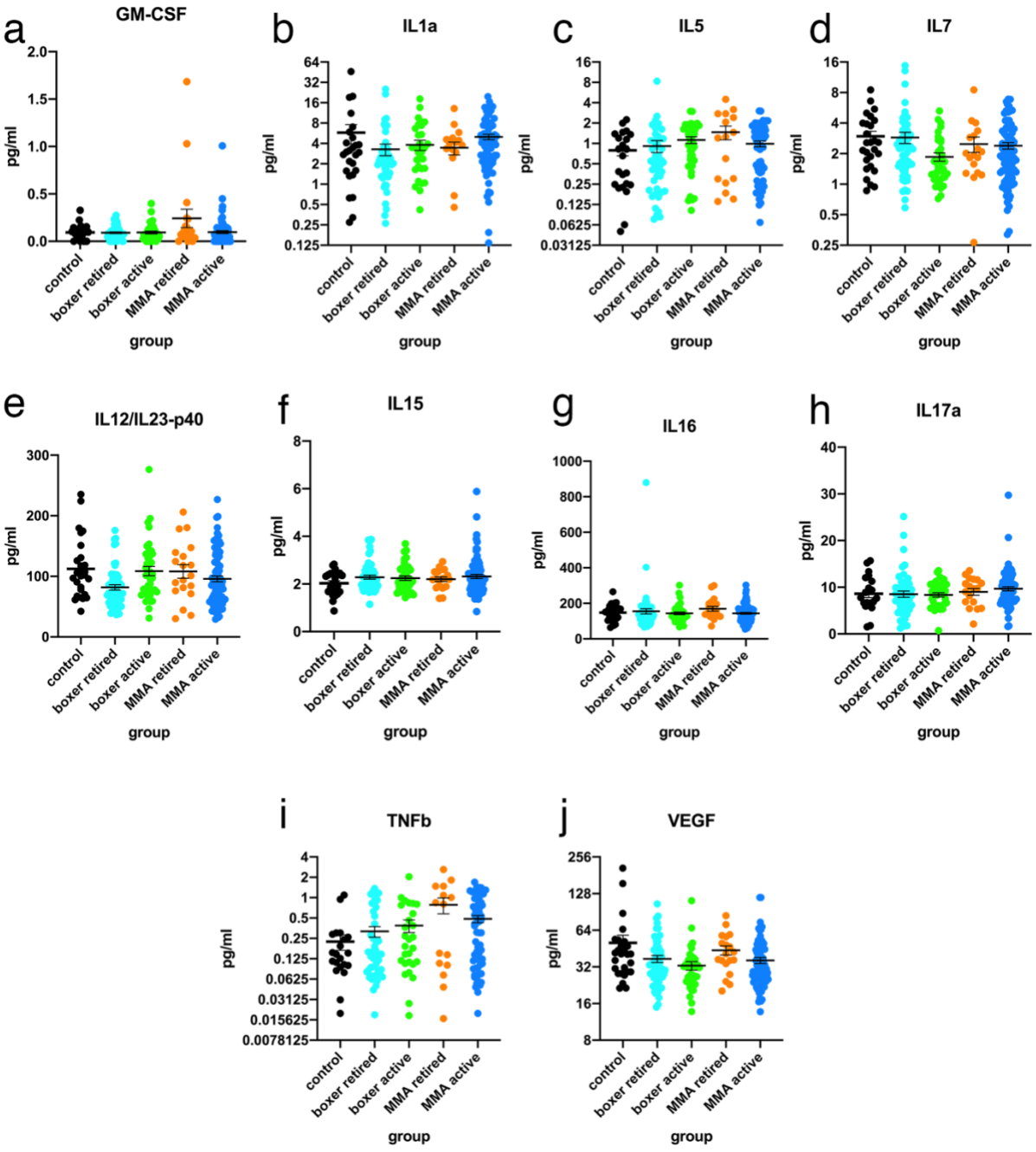

Figure 1. Plasma cytokine concentrations in active fighters, retired fighters and controls. The lines for each group represent the mean and the error bars represent 1 standard error of mean. Concentrations of GM-CSF (a), IL1α (b), IL5 (c), IL7 (d), IL12/IL23-p40 (e), IL15 (f), IL16 (g), IL17a (h), TNFβ (i), and VEGF (j) are plotted either on a linear or log2 graph to best show the range of values.

As expected, many of the analytes showed similar ranges in concentration amongst each group. Analytes showing similar concentrations included: IL1α, IL5, IL15, IL16,

and IL17a from the cytokine panel (**Fig 1**); CCL2, CCL22, CCL4, and eotaxin-3 from the chemokine panel (**Fig 2**); IL4, IL6, IL10, IL12p70, and IL13 from the proinflammatory cytokine panel (**Fig 3**); basic fibroblast growth factor (bFGF), PIGF, Tie-2, VEGF-D, and Flit1 from the angiogenesis panel (**Fig 4**); and ICAM1 from the vascular panel (**Fig 5**).

Differences in analyte concentrations will be described for each category. Those differences that were statistically different will be described with a *P* value. Some of the observations did not reach statistical significance, but will be described since the differences might be biologically relevant. One of the more striking observations was the differences in TNFβ (**Fig 1i**). The MMA retired cohort showed elevated levels compared to controls (0.783 pg/ml vs 0.224 pg/ml; *P* =

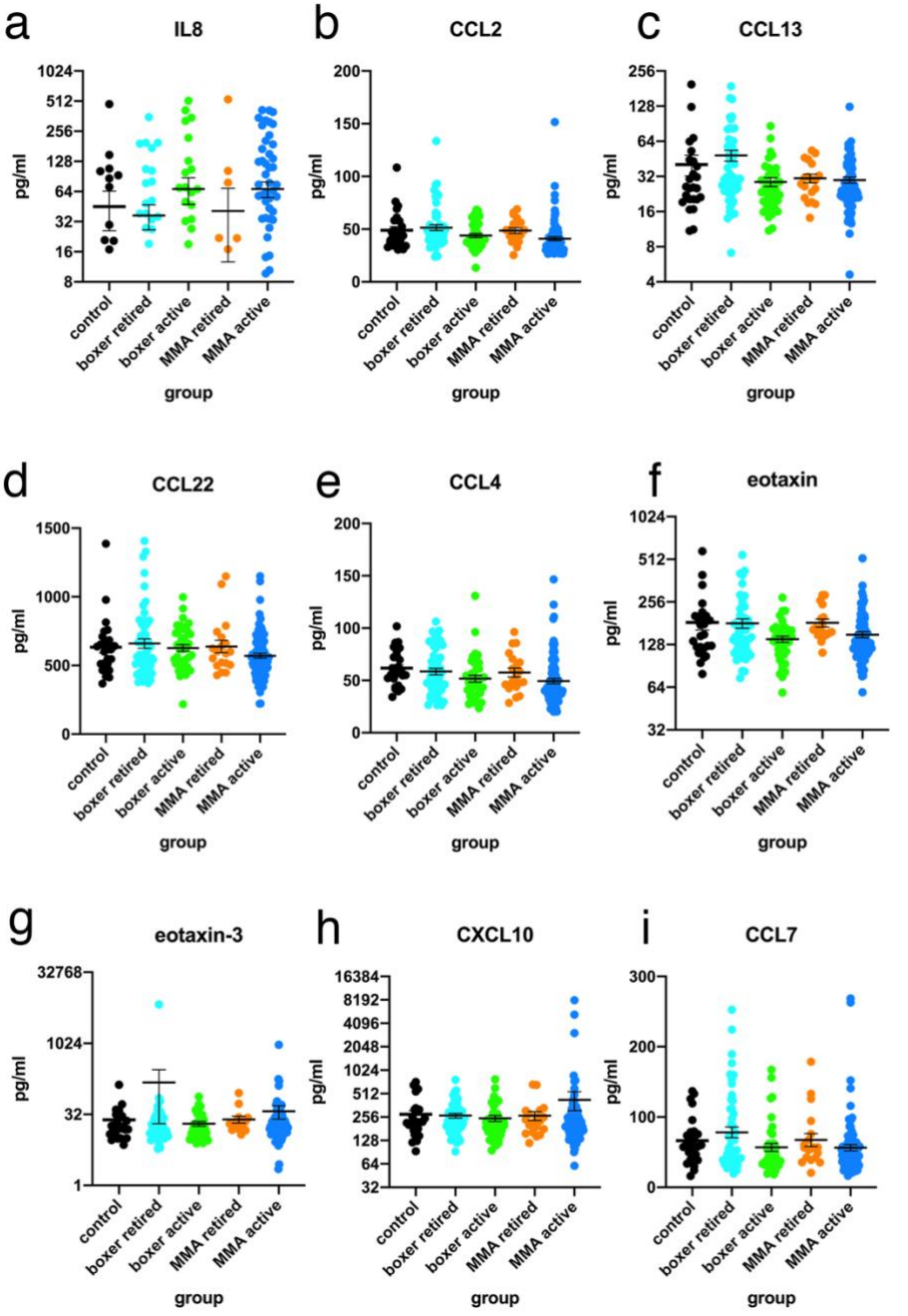

Figure 2. Plasma chemokine concentrations in active fighters, retired fighters and controls. The lines for each group represent the mean and the error bars represent 1 standard error of mean. Concentrations of IL8 (a), CCL2 (b), CCL13 (c), CCL22 (d), CCL4 (e), eotaxin (f), eotaxin-3 (g), CXCL10 (h), and CCL7 (i) are plotted either on a linear or log2 graph to best show the range of values.

0.004) and the Boxer retired cohort (0.783 pg/ml vs 0.317 pg/ml; *P* = 0.008). TNFβ was also elevated in the MMA active cohorts compared to controls. On the other hand, vascular endothelial growth factor (VEGF) was significantly reduced in both active boxers (32.754 pg/ml) and MMA fighters (36.184 pg/ml) compared to controls (50.373 pg/ml) (*P* = 0.01 and 0.03, respectively) (**Fig 1j**). Retired boxers showed decreased IL12/IL23-p40 concentrations (81.761 pg/ml) compared to controls (112.27 pg/ml) and active boxers (114.251 pg/ml) (*P* = 0.03 for both) (**Fig 1e**). IL7 concentrations were decreased in active boxers (**Fig 1d**). While GM-CSF concentrations were quite low in plasma, both the retired and active MMA fighters had individuals with elevated concentrations (**Fig 1a**). However, only the retired MMA fighters had statistically significant increases compared to all other cohorts (*P* = 0.02 – 0.009).

IL8 concentration measurements in the chemokine panel showed increased levels in both active boxers (67.68 pg/ml) and MMA fighters (67.73 pg/ml) compared to the other cohorts (**Fig 2a**), and this was statistically significant when compared to the MMA retired cohort (40.82 pg/ml) (*P* = 0.02 and 0.04). Retired boxers had higher concentrations of CCL13 (57.58 pg/ml) compared to active boxers (28.69 pg/ml) and MMA fighters (29.81 pg/ml) (*P* = 0.005 and 0.0009, respectively) (**Fig 2c**). Eotaxin levels were depressed in both active boxers and MMA fighters (**Fig 2f**). Interestingly, CXCL10 concentrations were elevated in some MMA fighters compared to other cohorts (**Fig 2h**), while CCL7 levels were depressed in active MMA fighters (56.44 pg/ml) compared to retired boxers (78.13 pg/ml) (*P* = 0.04) (**Fig 2i**).

MMA fighters likewise showed increased proinflammatory interferon γ levels compared to other cohorts **(Fig 3a**). There was a statistically significant increase in IL1β concentrations in retired versus active MMA fighters (*P* = 0.04), but this could be due to a relatively small number of retired MMA samples that were readable (**Fig 3b**). Intriguingly, IL2 concentrations were depressed in both retired (0.25 pg/ml; *P* = 0.003) and active (0.24 pg/ml; *P* = 0.005) boxers compared to controls (0.57 pg/ml) (**Fig 3c**). Surprisingly, TNFα levels were very similar among each cohort, although a few MMA fighters showed elevated concentrations (**Fig 3j**).

Angiogenesis protein concentrations were largely unaffected by history of boxing or MMA fighting. However, as observed in the cytokine panel, VEGF was decreased in active boxers and MMA fighters (**Fig 4d**). VEGF-C showed a similar trend as VEGF (**Fig 4e**).

Vascular markers showed an interesting tendency. Retired boxers showed elevated CRP levels (4.4 μg/ml) compared to other cohorts, with a statistically significant increase over active MMA fighters (1.54 μg/ml) (*P* = 0.03) (Fig 5a). Similarly, SAA was increased in retired boxers (Fig 5c). In contrast, VCAM1 levels we significantly decreased in retired boxers (0.38 μg/ml) compared to active MMA fighters (4.37 μg/ml; *P* = 0.03), retired MMA fighters, and active boxers (Fig 5d). However, retired boxers showed similar VCAM1 levels to controls (0.39 μg/ml).

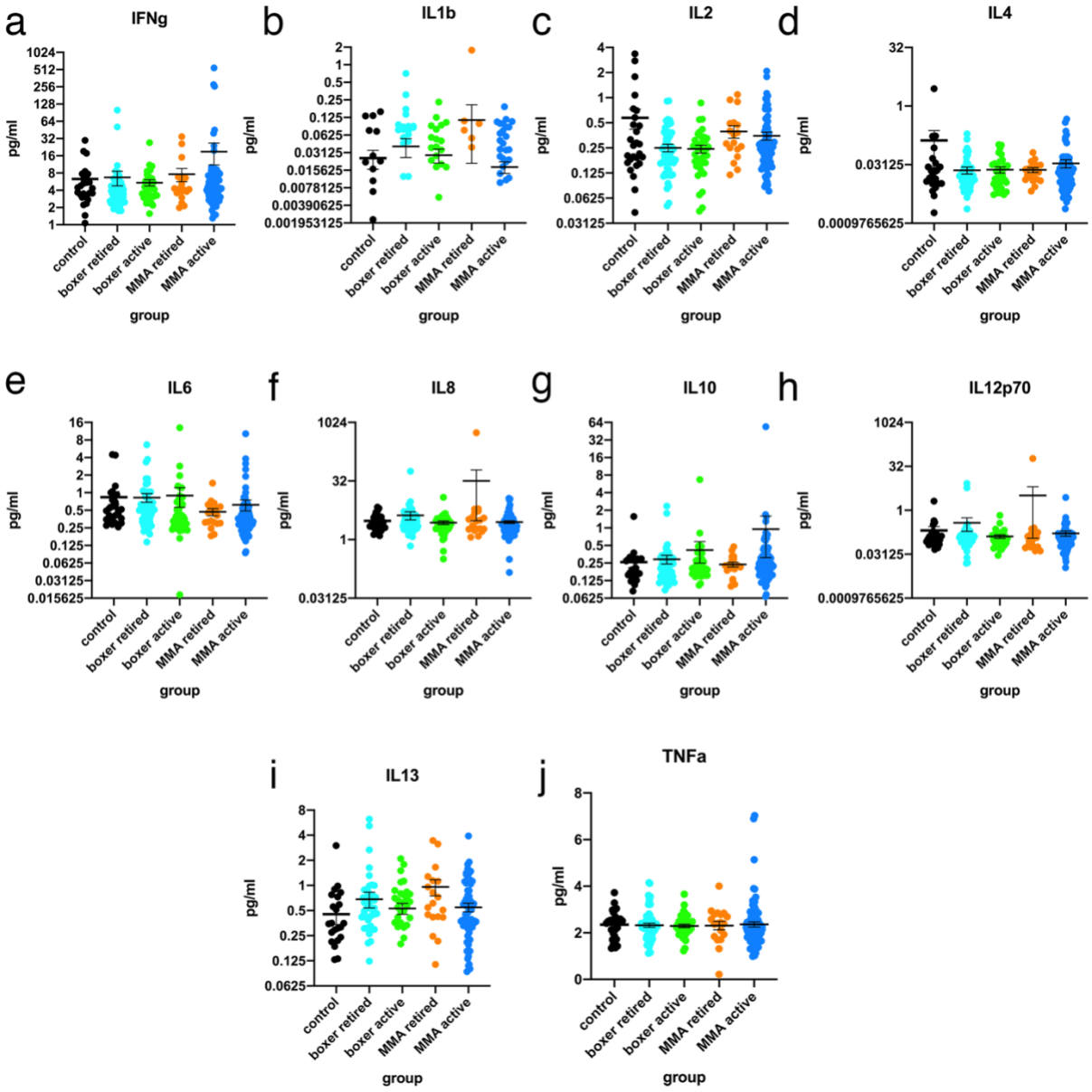

Figure 3. Plasma proinflammatory cytokine concentrations in active fighters, retired fighters and controls. The lines for each group represent the mean and the error bars represent 1 standard error of mean. Concentrations of interferon γ (IFNg) (a), IL1β (b), IL2 (c), IL4 (d), IL6 (e), IL8 (f), IL10 (g), IL12p70 (h), CCL7 (i) and TNFα (j) are plotted either on a linear or log2 graph to best show the range of values.

Two different antibodies recognizing IL8 were included in the analysis. The antibodies have differing sensitivities depending on the levels of IL8 present in a sample. The two different antibodies show slightly different results (**Fig 2a and 3f**). Since the results in Fig 2a are from the recommended antibody for higher levels of IL8, we believe this antibody would be most relevant; however, this would have to be validated in the future. Similarly, VEGF was represented in both the cytokine (**Fig 1j**) and angiogenesis panels (**Fig 4d**).

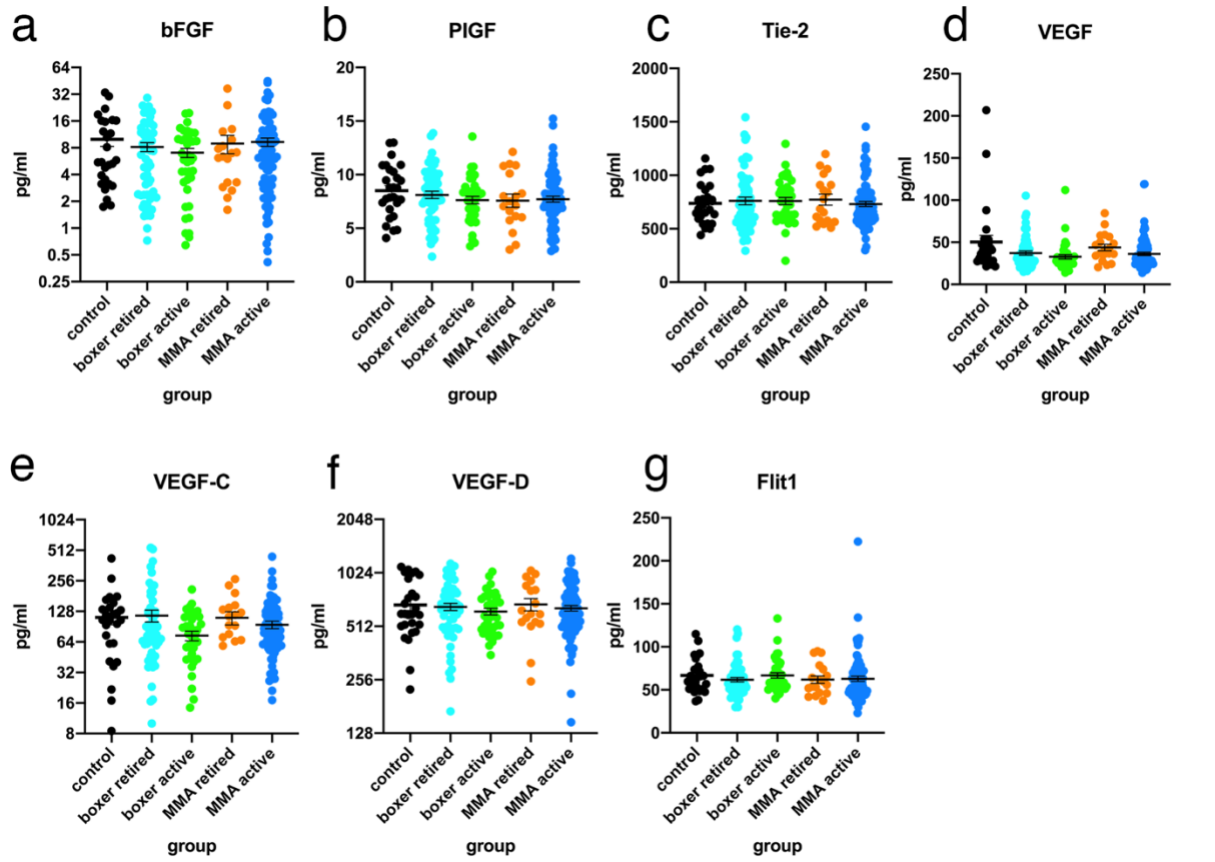

Figure 4. Plasma angiogenesis proteins in active fighters, retired fighters and controls. The lines for each group represent the mean and the error bars represent 1 standard error of mean. Concentrations of basic fibroblast growth factor (bFGF) (a), PIGF (b), Tie-2 (c), VEGF (d), VEGF-C (e), VEGF-D (f), and Flit1 (g) are plotted either on a linear or log2 graph to best show the range of values.

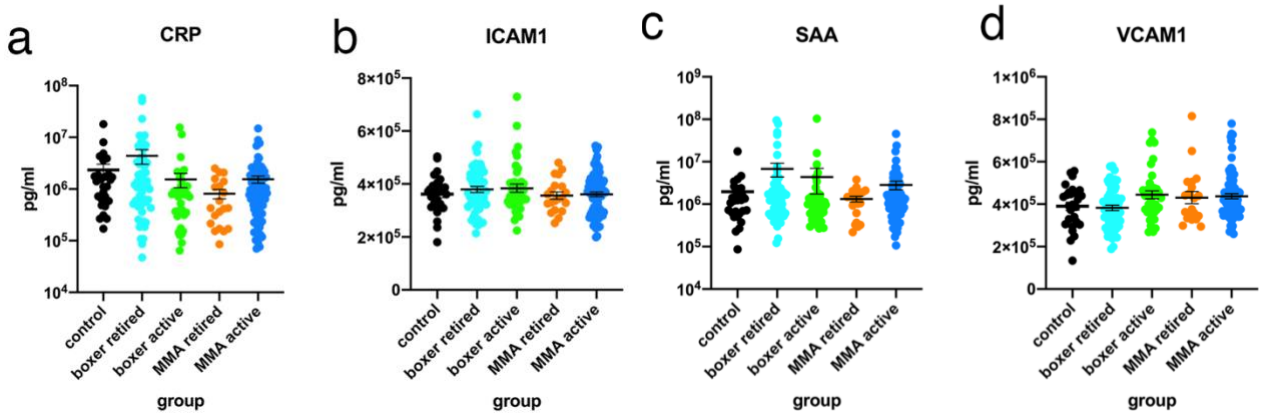

Figure 5. Plasma vascular proteins in active fighters, retired fighters and controls. The lines for each group represent the mean and the error bars represent 1 standard error of mean. Concentrations of CRP (a), ICAM1 (b), SAA (c), and VCAM1 (d) are plotted either on a linear or log2 graph to best show the range of values.

The detection antibody in both panels are the same. We observe similar VEGF concentrations and trends for each cohort, validating our results.

To address our hypothesis that TREM2 expression will be heightened in monocyte populations in the blood of active professional fighters compared to age/education-matched controls, single cell suspensions obtained from buffy coats were stained with antibodies recognizing CD45, CD14, CD16, and TREM2. Samples were subjected to flow cytometry, and data were processed and analyzed to obtain the percentage of CD45+CD14+, CD45+CD16+, CD45+CD14+CD16+, CD14+, CD16+, or CD45+ populations expressing TREM2. Data were also examined as a percentage of control. Fascinatingly, the data reveal that fighters have a lower proportion of monocytes expressing TREM2. All monocyte populations, including the CD14+CD16+ intermediate (CD45CD1416 on graph) and CD14+CD16dim (CD45CD14 on graph) inflammatory populations, showed significantly lower percentages of cells expressing surface TREM2 in both retired and active boxers (**Fig 6a**). The differences are striking when compared as a percentage of control (**Fig 6b**). The same observation was seen in active MMA fighters (**Fig 7**); compared to controls TREM2 expression on all monocyte populations was significantly lower.

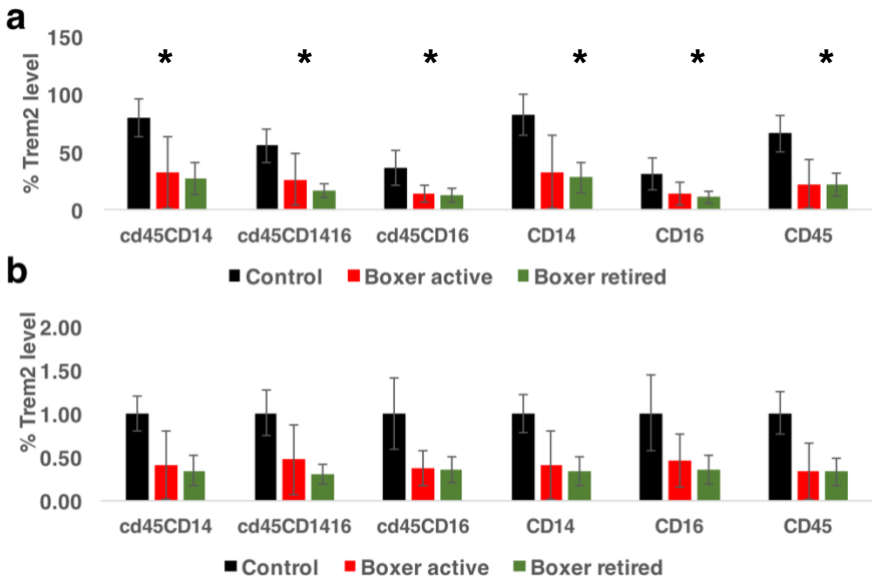

Figure 6. The proportion of TREM2 expressing monocyte populations in retired and active boxers is significantly lower than controls. (a) The percentage of CD45+CD14+, CD45+CD14+CD16+, CD45+CD16+ monocyte populations expressing TREM2 for control (black), active boxers (red) and retired boxers (green). Each bar represents the mean and standard error of the mean. ***** denotes statistically significant differences of both boxer active and boxer retired groups compared to controls as determined by ANOVA followed by Tukey's post hoc test for multiple comparisons. **(b)** Boxer active and Boxer retired groups as a proportion of the control group.

These findings indicate that TREM2 expression on circulating monocytes is impaired in both active and retired fighters, indicating that the deficiency is chronic. This striking finding has several implications for monocyte function and biology. It will be important to

interrogate whether this chronic deficiency of TREM2 expression alters monocyte trafficking to the brain and other organs, and how it affects the function of inflammatory and classical monocyte populations. We have observed that TREM2 expression is increased on monocyte populations in patients with Alzheimer's disease, so it will also be important to identify the inflammatory environment that promotes or diminishes TREM2 expression on monocytes and whether TREM2 expression on monocytes is beneficial or detrimental. TREM2 expression may be subject to disease stage-specific regulation. On the same vein, we have begun to dissect the inflammatory environment found in active and retired fighter plasma. We see that active fighters (both boxers and MMA fighters) have altered concentrations of TNFβ, IL8, and eotaxin, indicating an acute response. Both retired and active MMA fighters showed changes in GM-CSF, CXCL10, CCL7, and IFNγ concentrations. Retired boxers showed altered CRP and SAA levels, potentially indicating a chronic response to previous professional fighting. Finally, VEGF and VEGF-C concentrations were decreased in both active and retired fighters. This is interesting since cellular responses to VEGF stimulus are enriched differentially expressed genes in TREM2-deficient mice (Carbajosa et al, Neurobiology of Disease, 2018, Vol 69:151), suggesting a potential relationship between VEGF and TREM2 expression.

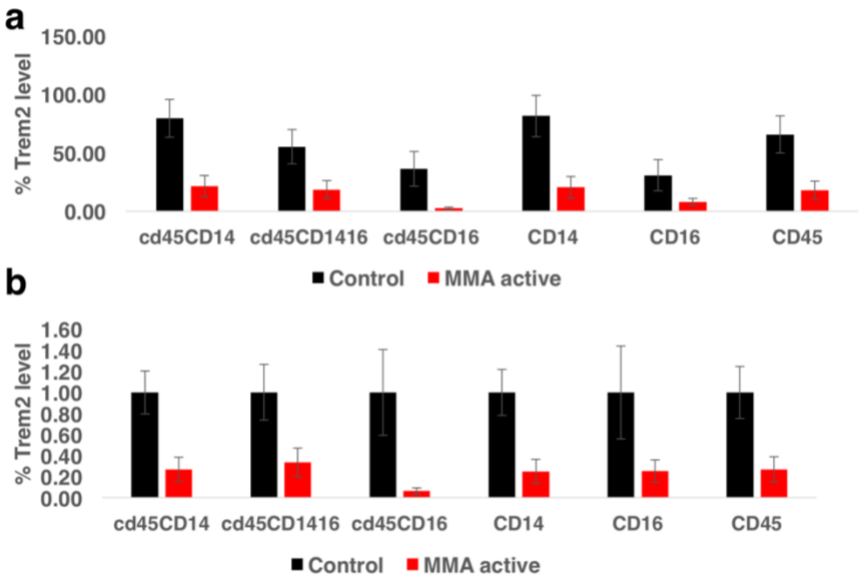

Figure 7. The proportion of TREM2 expressing monocyte populations in active MMA fighters is significantly lower than controls. (a) The percentage of CD45+CD14+, CD45+CD14+CD16+, CD45+CD16+ monocyte populations expressing TREM2 for control (black) and active MMA fighters (red). Each bar represents the mean and standard error of the mean. ***** denotes statistically significant differences of both boxer active and boxer retired groups compared to controls as determined by ANOVA followed by Tukey's post hoc test for multiple comparisons. **(b)** MMA active cohort as a proportion of the control cohort.

What opportunities for training and professional development have the project provided?

This project has provided the trainees multiple opportunities for professional development. Drs. Shane Bemiller and Nipun Chopra attended the Society for Neurosciences Local Chapter Conference in Indianapolis, IN in May 2017.

Salary support was granted for Dr. Chopra, who was trained to utilize flow cytometry. Dr. Chopra used his experience on this project to advance his career as an Assistant Professor of Biology at Depauw University in Greencastle, IN.

Dr. Shane Bemiller was able to visit the Las Vegas study sites at UNLV and CCF in 2017. There, he spearheaded setting up and training Dr. Kinney's lab to perform the TREM2 expression analysis on monocyte populations using flow cytometry. Dr. Bemiller took the experience gained in setting up biomarker studies to advance his career as a Field Applications Scientist at MESO SCALE DIAGNOSTICS.

Dr. Jefferson Kinney has added a new expertise to his laboratory. Dr. Kinney's group, trained by Dr. Bemiller, is now proficient in the analysis of cell surface marker expression using flow cytometry. This will enable him to expand his studies in analyzing novel biomarkers in traumatic brain injury, Parkinson's disease, and Alzheimer's disease.

Mr. McCray, a graduate student in the Medical Neuroscience program at Indiana University School of Medicine, was instrumental in the analysis of plasma biomarkers. He is currently involved in writing the results of this study for publication, which will further his career.

Ultimately, these experiences served as a platform to disseminate results and to discuss data generated under this award.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Future directions of project include submission of two manuscripts. The first manuscript is currently in preparation by the Kinney lab and will describe the deficiency of TREM2 expression on monocytes. The second manuscript will describe the plasma biomarker analysis and will be spearheaded by Tyler McCray and Dr. Stephanie Bissel from the Lamb lab. We plan to consult with the biostaticians from the Department of Biostatistics at the IU School of Medicine that offer support services in order to confidently control for covariates such as age, race and education in our analysis. Publishing of these manuscripts will disseminate the results to communities of interest. Finally, to wrap up this study, we will upload our data to the FITBIR system later this month (November 2020).

Given the intriguing findings of this study, we plan to submit an application to explore the biology of TREM2 expression on monocytes and how its absence might influence neurodegenerative outcomes in repetitive head injury. This application is in the nascent stage of development; however, the data described in this report will be the basis of the application. We will address diagnostic biomarker potential and potential therapeutic manipulation. We will also address whether other forms of traumatic brain injury experienced by warfighters (relatively acute), veterans (chronic), and accidents experienced by the general population will result in the same deficiency in TREM2 expression on monocytes. It will be interesting to see changes in our markers of interest over time.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Cerebral inflammation is an acute hallmark feature of TBI that can promote recovery if controlled; however, excessive or sustained inflammation has been linked to progressive changes in the brain including atrophy, neuronal loss, and axonal degeneration. The inflammatory response following TBI is thought to induce pathological events that predispose individuals with brain injury to develop AD-related pathology later in life. Accumulating experimental evidence indicates that repetitive brain injury promotes a persistent inflammatory response at chronic post-injury time points. One goal of the project was to characterize the acute inflammatory response to repetitive head trauma in active professional fighters. We found that active fighters (both boxers and MMA fighters) have altered concentrations of TNFβ, IL8, and eotaxin in their plasma. This adds to reports indicating that neurofilament-light chain is increased in the plasma of active fighters, reflecting acute exposure. The second goal was to characterize the chronic inflammatory response to repetitive head trauma in retired professional fighters that should have infrequent head injuries. Retired boxers showed altered CRP and SAA levels, potentially indicating a chronic response to previous professional fighting. This adds to reports showing that changes in plasma tau levels reflect chronic change in retired fighters who show changes in brain structure. Some acute changes in the inflammation plasma markers of active fighters also persist in the retired fighters including
GM-CSF, CXCL10, CCL7, IFNγ, VEGF and VEGF-C concentrations. These results indicate that the inflammatory environment shows some difference between acute and chronic repetitive head injury; however, some alterations persist chronically.

Likewise, peripheral blood monocytes showed impaired TREM2 expression in both active and retired fighters, indicating that the deficiency is chronic. Interestingly, this is contrasts with our original hypothesis that TREM2 expression on monocytes in active and retired fighters will be increased. TREM2 has been shown to be involved in multiple various inflammatory diseases; however, the exact role of TREM2 is not yet known. Multiple studies looking at the role of TREM2 in disease states, both *in vitro* and *in vivo*, have shown contradictory results. These results have suggested that the role of TREM2 may

be different in different pathological contexts. One goal of this project was to study the role of TREM2 in the context of TBI experienced by active and retired professional fighters (boxers and MMA fighters) and determine whether it contributes to neurodegeneration and Alzheimer's disease pathology. In a separate study, we have observed that TREM2 expression is increased in patients with Alzheimer's disease, so it will also be important to identify the inflammatory environment that promotes or diminishes TREM2 expression on monocytes and whether TREM2 expression on monocytes is beneficial or detrimental. TREM2 expression may be subject to disease stage-specific regulation. These studies highlight the complexity of identifying therapeutic targets that have different functions in different pathological contexts. It will be important to dissect the biology in the future. Finally, changes observed in the retired fighters correlate with changes in brain structure. It will be important to determine the role of the inflammation-related alterations we have observed in promoting these changes that are thought to predispose fighters to Alzheimer's disease.

What was the impact on other disciplines?

These findings impact not only the TBI field, but the immunology and neurodegeneration fields as well. The role of TREM2 has been canonically thought to be anti-inflammatory in the immunology field. However, in the neurodegeneration field, the function of TREM2 has been controversial with contradictory findings. Showing that TREM2 expression on peripheral blood monocytes is chronically impaired after repetitive head injury while it is increased during Alzheimer's disease is striking. This highlights the complexity of TREM2 biology but concurrently adds more keys to finding what TREM2 does in different pathological contexts in acute and chronic disease and how it affects neurodegeneration.

What was the impact on technology transfer?
Nothing significant to report.

What was the impact on society beyond science and technology?

These results support the hypothesis that persistent immune changes are detrimental to the aging/neurodegeneration process. Participation in activities where one experiences repetitive head trauma lead to persistent alterations in inflammation-related processes. Initiatives to prevent brain injury, dampen inflammatory processes through life style choices (e.g. exercise and diet), and prevent stress could have a dramatic impact on neurodegenerative disease incidence and progression.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

This study was plagued with relocation and personnel changes. After the move to Indiana, we were considerably delayed in completing the goals of the grant. We were graciously granted an extension to perform our studies. In Indiana, personnel

spearheading the project for the Lamb lab changed dramatically and often. Dr. Atsuko Katsumoto was originally directing this project. She left shortly after the move to Indiana when Dr. Shane Bemiller took over. Dr. Bemiller made many important strides to get the project back on track including training the Kinney lab to perform the flow cytometry analysis. Unfortunately, Dr. Bemiller left in early 2018 leaving Dr. Nipun Chopra in to direct the project. Dr. Chopra's tenure was short, leaving in 2019. Dr. Stephanie Bissel then was positioned to lead the project. She organized approvals, help facilitate the shipment of samples, and identified Tyler McCray to perform the plasma biomarker analysis in efforts to complete the goals of the study. The plasma biomarker study was on track to be finished in early March of 2020 when the COVID shutdowns occurred. We finally were able to collect all of the data for analysis.

Dr. Jefferson Kinney's participation had been on autopilot since Dr. Bemiller trained his lab. Fortunately, all his samples were run before the COVID shutdowns.

Communication with the Luo Ruvo Center for Brain Health (LRCBH) at the Cleveland Clinic in Las Vegas, Nevada was difficult for most of the project and even more so during the last 1-2 years. This was due to extreme personnel turnover. We were able to obtain the fresh blood samples and frozen plasma aliquots as well as the PHI for each subject from LRCBH. LRCBH had difficulty in recruiting subjects for participation in the CSF, so we were not able to obtain sufficient numbers of samples to make meaningful analysis. Likewise, the MRI and PET scans were presumably performed, but due to the turmoil at LRCBH, we have not been able to obtain the data for inclusion in this project at the time of this report. These scans were not supported through the funds allocated to this project as there were part of the parent PFBHS.

Actual or anticipated problems or delays and actions or plans to resolve them

The remaining action items to complete this grant include publication of the results. Dr. Bissel and Mr. McCray have begun writing the manuscript characterizing the plasma biomarkers in active and retired fighters and plan to submit by the end of the year. Dr. Kinney has been writing the manuscript characterizing TREM2 expression on peripheral blood monocytes and also plans to submit the manuscript shortly.

Changes that had a significant impact on expenditures
Due to all of the delays, several extensions were requested to complete the goals of the project, but we were able to complete the studies with the original budget.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing significant to report.

Significant changes in use or care of human subjects
Not applicable.

Significant changes in use or care of vertebrate animals.
Nothing significant to report.

Significant changes in use of biohazards and/or select agents.
Nothing significant to report.

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications

- *Bemiller SM, Mourany L, Jay T, Cheng P, Cotleur A, Formica S, Xu G, Lee M, Ransohoff RM, Rao S, Pillai J, Bekris L, Landreth GE, Leverenz J, Lamb BT. Peripheral TREM2 in Alzheimer's Disease (Under review *JCI 2018*). * This was under review at JCI when Dr. Bemiller left the lab. No one was left to lead the response. There are plans to resurrect the publication of the manuscript.

Books or non-periodical, one-time publications
Nothing to Report.

Other publications, conference papers, and presentations
Abstracts

- Peripheral Roles for TREM2 and CD33 in Alzheiemer's Disease. Bemiller SM., Ransohoff R., Bekris L., Leverenz J., Saykin, A, Lamb BT. SFN Greater Indianapolis Chapter Anniversary Symposium, November 11th 2016, Indianapolis, IN.

Seminars

- Bemiller, SM. The role of TREM2 in regulating Alzheimer's Disease pathology. January 2017. Invited talk at Ashland University, Ashland, Ohio.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Invention, patent applications, and/or licenses
Nothing to report.

Other products
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Bruce T. Lamb
Project Role:	Principal Investigator
Researcher Identifier:	
Nearest person month worked:	1
Contribution to Project:	Dr. Lamb was the lead principal investigator on this project. He provided expertise in experimental design, data analysis and interpretation of results.
Funding Support	National Institutes of Health, BrightFocus Foundation, Alzheimer's Association, Department of Defense, Indiana University

Name:	Jefferson Kinney
Project Role:	Principal Investigator
Researcher Identifier:	
	6
Contribution to Project:	Dr. Kinney and his group isolated blood cells and performed flow cytometry analysis. He was also involved in data analysis, interpretation and preparing the manuscript for publication.
Funding Support	National Institutes of Health, Department of Defense

Name:	Sarah Banks
Project Role:	Co-Investigator
Researcher Identifier:	
Nearest person month worked:	1
Contribution to Project:	Dr. Banks worked with the Bernick group to facilitate sample transfers and imaging. She was involved with the project until 2018.

Name:	Shane Bemiller
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	9

Contribution to Project:	Dr. Bemiller was the project manager coordinating efforts between IU and UNLV/CCF. He trained the Kinney lab to perform the flow cytometry analysis. He was involved in the project from 2016 to 2018.

Name:	Nipun Chopra
Project Role:	Postdoctoral fellow
Researcher Identifier:	
Nearest person month worked:	6
Contribution to Project:	Dr. Chopra first assisted with data collection and interpretation. He then became the project manager after Dr. Bemiller left in 2018. He was involved with the project from 2017 to 2019.

Name:	Pamela Dino
Project Role:	Patient/Sample Coordinator
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	Ms. Dino works with the Bernick group to coordinate subject participation, data collection, and sample distribution.

Name:	Tyler McCray
Project Role:	Research Technician
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	Mr. McCray performed the plasma biomarker analysis. He was involved in the project from 2019-2020.

Name:	Stephanie Bissel
Project Role:	Assistant Research Professor
Researcher Identifier:	
Nearest person month worked:	4
Contribution to Project:	Dr. Bissel started as the project manager in 2018, coordinating efforts between IU, UNLV, and CCF. She was also involved in data analysis, interpretation and preparing the manuscript for publication.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Active Other Support for Bruce T. Lamb

R01AG022304 (Rao) 09/01/17-05/31/22 2.40
CM
Cleveland Clinic Lerner College of Medicine of CWRU (NIH pass-through)
Immune Mechanisms Underlying the Neuroprotective Effects of Physical Activity in Human and Mouse Models of Genetic Risk for Alzheimer's Disease
Dr. Bruce Lamb's laboratory at Indiana University School of Medicine will conduct the studies described in Specific Aim 2, including all of the animal work including all of the biochemical, flow cytometry, histological and behavioral studies proposed. Dr. Lamb will also provide guidance on the design of the studies and will play an integral role in the interpretation of results from the studies across of aims of the proposed project. (Role: Subcontract PI and Co-Investigator)

P30 AG010133 (Saykin) 07/01/16-06/30/21 0.84
CM
NIH-NIA
Indiana Alzheimer Disease Center
The major goal of this project is to support, carry out and facilitate research on Alzheimer disease and other neurodegenerative dementias as well as serving as a shared research resource.
Roles: Co-I on Neuropathology Core at 0.24 CM and Co-I on Research Education Core at 0.60 CM for a total of 0.84 CM.

U54AG054345 (Lamb, contact PI; Territo/Howell/Carter MPI) 09/30/16-08/31/21 1.80
CM
NIH-NIA
04 Award
NIH/NIA – supplement 03S6 (PTC Transition)
NIH/NIA – supplement 04S1 (Aging)
NIH/NIA – supplement 04S2 (Microbiome)
The IU/JAX Alzheimer's Disease Precision Models Center
The interdisciplinary Indiana University/Jackson Laboratory Alzheimer's Disease Precision Models Center (IU/JAX ADPMC) seeks to generate and characterize novel animal models of AD, assess the relevance of these to model to human disease and to develop a preclinical testing pipeline through which novel therapies can be tested to greatly accelerate the process by which therapies are successfully moved forward to human AD clinical trials. (Role: PI)

RF1AG050597 (Landreth, contact PI; Lamb PI) 02/01/17 - 03/31/21 0.60
CM
NIH/NIA
Actions of Nuclear Receptors on TREM2+ Myeloid Cells and Microglia in the AD Brain
The Lamb and Landreth labs will collaborate on the generation of contemporary genetic mouse models that selectively express fluorescent markers in blood borne inflammatory monocytes to definitively establish the peripheral origins of plaque-associated cells in a mouse model of Alzheimer's disease. (Role: PI)

RF1AG051495 (Lamb, contact PI; Landreth PI) 05/15/17 – 08/31/20 2.40 CM
NIH/NIA
Central and Peripheral Roles of TREM2 in Alzheimer's Disease
To examine whether brain resident microglia and blood-derived TREM2+ monocytes play distinctive roles in regulating Aβ pathology and whether the TREM2+ cell population could provide novel biomarkers/diagnostics and be targeted therapeutically. (Role PI)

U54AG065181 (Palkowitz, contact PI; Lamb PI) 09/30/19-08/31/24 1.20 CM
NIH-NIA
IUSM Alzheimer's Disease Drug Discovery Center
The strategic goal of the Indiana University School of Medicine Alzheimer's Disease Drug Discovery
(ADDD) Center is to integrate sophisticated capability for early drug discovery and contribute to a more broad
study of emerging Alzheimer's Disease target hypotheses (beyond Aβ) with the goal of generating new classes
of potential therapeutics. (Role: PI)

What other organizations were involved as partners?
Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards
Nothing to Report

Quad Charts
Nothing to Report

9. APPENDICES

Peripheral Roles for TREM2 and CD33 in Alzheiemer's Disease.

Bemiller SM., Ransohoff R., Bekris L., Leverenz J., Saykin, A, Lamb BT.

SFN Greater Indianapolis Chapter Anniversary Symposium, November 11th 2016, Indianapolis, IN.

Recently identified coding variants of innate immune regulating genes Triggering Receptor Expressed on Myeloid Cells-2 (TREM2) and Cluster of Differentiation-33 (CD33) confer increased risk of developing late-onset Alzheimer's Disease. Using flow cytometry, we have characterized levels of TREM2 protein on classically defined subsets of human monocytes and demonstrated increased percentages of CD14+CD16dimTREM2+ inflammatory monocytes in AD patients compared to healthy control individuals. Significant inverse correlations were found between monocyte TREM2 expression and CSF levels of total tau and phospho-tau. Further, CD33 protein levels on intermediate and patrolling monocyte populations were strongly associated with levels of CSF Aβand cognitive measures. These findings suggest role for the peripheral innate immune system in the pathogenesis of Alzheimer's disease.