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Microarray analysis of mouse ling examining the augmented pseudomonas aeruginosa clearance following mild traumatic brain injury

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MICROARRAY ANALYSIS OF MOUSE LUNG EXAMINING THE AUGMENTED PSEUDOMONAS AERUGINOSA CLEARANCE FOLLOWING MILD TRAUMATIC BRAIN INJURY

by

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MICROARRAY ANALYSIS OF MOUSE LUNG EXAMINING THE AUGMENTED PSEUDOMONAS AERUGINOSA CLEARANCE FOLLOWING MILD TRAUMATIC BRAIN INJURY

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ABSTRACT

Our murine model of mild traumatic brain injury (mTBI) has shown improved survival after *Pseudomonas aeruginosa* (*Psd*) challenge as compared to controls (tail trauma or sham injury). Previous work suggests an mTBI-specific involvement of the neuro-immune axis which augments the innate immune response, increasing survival. Additional factors for the enhanced mTBI survival were explored via microarray analysis of lungs harvested 48 hours post-trauma, the point prior to *Psd* challenge in our model. At 48 hours post-trauma, mTBI lungs have a number of upregulated ATP synthesis and mitochondrial gene sets. Increased available energy could prime the mTBI lungs, allowing an earlier and more robust response to *Psd* infection, possibly contributing to the increased mTBI survival. This is supported by increased neutrophil recruitment in the bronchoalveolar lavage of mTBI mice four hours after *Psd* instillation. Downregulated gene sets related to cellular connections suggest that neutrophils recruited to the lung have an easier extravasation pathway into the air space of mTBI lungs compared to control. Based on genetic and neutrophil recruitment data, it is possible that mTBI creates an energetically prepared and easily accessible lung better tailored for recruiting and allowing entry of neutrophils in response to an infection compared to control.

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LIST OF ABBREVIATIONS

ATP ..... Adenosine Triphosphate

BAL ..... Bronchoalveolar Lavage

cDNA ..... Complementary Deoxyribonucleic Acid

CFU ..... Colony Forming Units

DAMP ..... Damage-associated Molecular Pattern

DNA ..... Deoxyribonucleic Acid

eIF ..... Eukaryotic initiation factor

GO ..... Gene Ontology

GSEA ..... Gene Set Enrichment Analysis

GTP ..... Guanosine Triphosphate

KEGG ..... Kyoto Encyclopedia of Genes and Genomes

mRNA ..... Messenger Ribonucleic Acid

MSigDB ..... Molecular Signatures Database

mTBI ..... Mild Traumatic Brain Injury

NF-κB ..... Nuclear Factor Kappa-light-chain-enhancer of Activated B cells

NK1R ..... Neurokinin 1 Receptor

NUSE ..... Normalized Unscaled Standard Error

PABP ..... Poly(A)-binding Protein

PC ..... Principal Component

PCA ..... Principal Component Analysis

PCR ..... Polymerase Chain Reaction

PMN ..... Polymorphonuclear Leukocyte

Psd ..... *Pseudomonas aeruginosa*

RLE ..... Relative Log Expression

RNA ..... Ribonucleic Acid

ROS ..... Reactive Oxygen Species

SP ..... Substance P

TBI ..... Traumatic Brain Injury

tRNA ..... Transfer Ribonucleic Acid

TT ..... Tail Trauma

INTRODUCTION

Traumatic brain injury (TBI) is a serious global health issue responsible for close to 2.5 million emergency department visits per year [1]. A TBI can trigger a series of complicated physiological events which have repercussions far beyond the initial injury, often causing permanent alterations in the brain and increasing the incidence of chronic pathological diseases such as seizures, sleep disorders, and psychiatric diseases, amongst other complications[2]. However, the full range of the repercussions of a TBI, and their mechanisms, are still relatively unknown. There are three levels of TBI: mild, moderate, and severe. Our research is focused on mild traumatic brain injury (mTBI) as it is the most common TBI in humans[3]. mTBI includes concussions and sub concussive forces and even though mTBI is the least severe of TBIs, it still results in a "neurometabolic cascade of concussion"[4] which releases neurotransmitters and causes fluctuations in ionic balances. In addition to the cerebral metabolic alterations, inflammatory and immunological responses are stimulated[5]. Following injury, recruitment of immune and glial cells to the site of trauma is increased by upregulation of the cell adhesion molecules P-selectin, ICAM-1, and VCAM-1[5]. The local infiltration of leukocytes is aided by the release of proinflammatory cytokines like TNF, IL-1β, and IL-6 shortly after injury[6]. However, these cytokines can initiate a cascade of events downstream of the mTBI, resulting in systemic alterations in the organism.

While any TBI may be harmful to the general public, further analysis of the downstream mTBI consequences suggests that not all physiological responses may be detrimental, as evidenced by Yang et al. (2010). Amongst patients hospitalized for non-

head trauma or mTBI who developed pneumonia, the mTBI patients showed increased survival[7]. This interesting clinical observation initiated the development of a murine model of mTBI to further investigate the novel survival findings and attempt to replicate them[7]. The mouse model of mTBI developed in our laboratory, compared to a control blunt trauma localized to the tail, was able to replicate the same increased survival to pneumonia of subjects hospitalized with brain trauma versus blunt trauma as was observed in the human cohort[7, 8]. This mouse model of mTBI is the model used in this thesis. Combined with the increased survival of mTBI mice, there was increased recruitment of polymorphonuclear leukocytes (PMN) to the lung space[7] as well as enhanced killing of bacteria by the immune system of the mTBI mice compared to the control tail trauma (TT) mice. The increased survival and enhanced innate immune response of the mTBI mice compared to the TT mice when challenged with pneumonia raises questions as to the mechanism responsible. Initial analyses of the counterintuitive results are thought to be related to the neuropeptide Substance P (SP).

Previous studies[8,7] suggest that SP, an eleven amino acid neuropeptide which acts through the tachykinin neurokinin-1 receptor (NK1R), plays an important role in augmenting the immune response after mTBI. It is believed that following mTBI, SP is released from the site of injury and acts on the NK1Rs throughout the organism. Other neurokinin receptors exist (NK2R and NK3R), and depending on concentrations of SP and receptor availability, SP can act through them. However, SP acts preferentially on NK1R[9]. SP is known to recruit inflammatory cells into tissues either through its effect on the tissue's NK1Rs (vasodilation) or the inflammatory cell's NK1Rs (chemotaxis)[10]. Our

interest in SP for our mTBI model is focused on its chemotactic properties on neutrophils and other immune responders, which are well documented[11,12,13]. The hypothesis for the role of released SP from the mTBI acting on the NK1Rs throughout the mouse to enhance survival and immune response is supported by NK1R agonist and antagonist studies[8]. When the mTBI mice were treated with an NK1R antagonist, then challenged with bacteria, the increased survival effect was not seen, supporting the importance of SP as a principal mediator of the increased survival of the mTBI mice.

While previous studies in our lab explore the role of SP in this augmented immune response, this study will examine the genetic changes induced in the lung after mTBI which could contribute to the augmented immune response and increased survival. The design of the current study is as follows (Figure 1), mice receive mTBI or TT and 48 hours later, are sacrificed. The lungs are removed and homogenized so RNA can be extracted. The RNA is then run on a microarray chip for analysis of genetic changes. The RNA of the lung is analyzed at 48 hours for two reasons. First, 48 hours is the usual timeline of pneumonia development in the human cohort of previous studies. Second, our previously published work administered *Pseudomonas aeruginosa* (*Psd*) 48 hours after mTBI or TT. The purpose of this study is to identify any possible differences in the genetic landscape of the site of infection between mTBI and TT at the point immediately prior to the pathogenic insult.

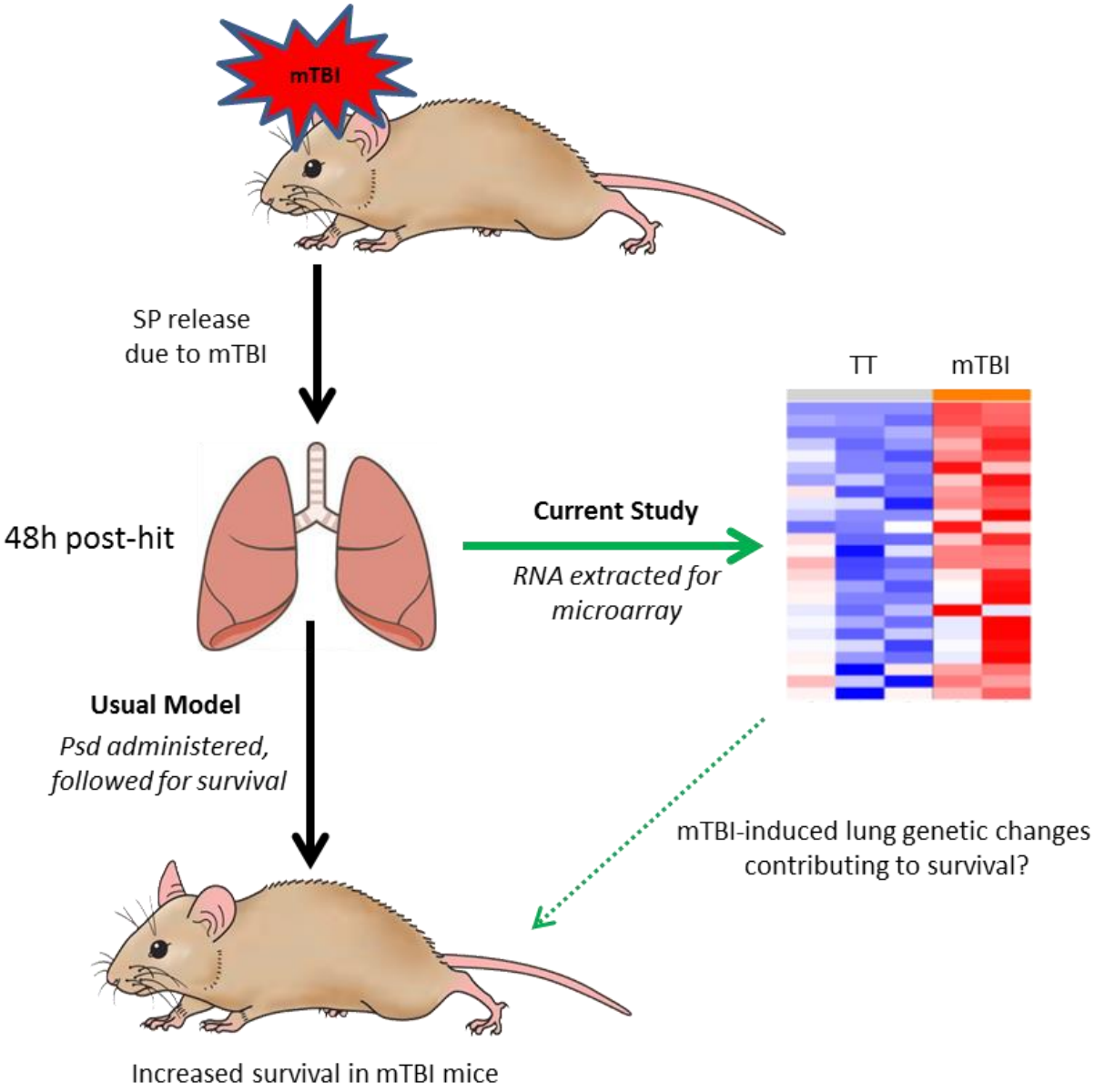

Figure 1: Design of current study. Mice receive mTBI (n=2) or TT control (n=3) and 48 hours later, at the time point mice usually receive *Psd*, lungs are harvested and RNA is extracted for microarray analysis. This is done to determine any genetic influences which could contribute to the usual model's results. We hypothesize that the changes in the gene expression of mTBI lungs could contribute to the increased survival of the mTBI mice when they receive *Psd* 48 hours after trauma in our usual model.

METHODS

Animals

Female ICR (CD-1) mice (Harlan Laboratories, Inc., Frederick, MD) were used in all experiments. Mice could access food and water *ad libitum* for the entire study. Animals were housed in a room set on a diurnal 12 hour light-dark cycle that was temperature and humidity controlled. They were allowed to acclimate for at least 72 hours before any experiments. All mice were 24-30g and at least 8-10 weeks old. All experiments were approved by the Boston University Institutional Animal Care and Use Committee.

Trauma model

Mild traumatic brain injury (mTBI) was performed as previously described[7]. Under isoflurane anesthesia, mice were placed prone on a Plexiglas bed with their head resting on a foam pad under a 170g steel rod encased in a guide tube. The rod is dropped from 5.2 cm above the skull to impact a point in the midline of the skull, producing a 5kg/cm^2 impact force halfway between the interauricular and interorbital lines. Rebound impact is prevented and mice were immediately injected subcutaneously with 0.05 mg/kg buprenorphine in 1 ml of warmed normal saline. Mice were placed supine on a warming bed until recovered and able to right themselves. The control trauma model, Tail Trauma (TT), was performed as previously described [8]. TT was induced by placing isoflurane-anesthetized mice in a prone position under the weight drop mTBI apparatus. The 170g rod was dropped from a height of 8.5 cm on the tail 2 cm from the base. TT mice

received the same buprenorphine dose and were placed on the warming bed before returning to their cages after righting themselves.

RNA Extraction

Mice were anesthetized with isoflurane, xylazine/ketamine and exsanguinated via retro-orbital bleeding. All mice underwent bronchoalveolar lavage (BAL) with 1 ml of a 50:1 HBSS/EDTA mixture (169 mM EDTA). The lavage fluid was spun and supernatant was frozen for later analysis. After BAL, two random sections of the right lung were sampled from the superior and inferior lobe of each mouse to introduce randomization and heterogeneity of the tissue sample collected. The left lung was perfused with formalin and removed for later histology. The right lung sections were stored in RNAlater (Thermo Fisher Scientific, Waltham, MA) overnight. After RNAlater fixation, a section from each of the two lung samples were placed in 600 μL of buffer RLT (Qiagen, Hilden, Germany) with 6 μL of 14.3 M beta-mercaptoethanol (Sigma) then homogenized using the TissueLyser II at 30 Hz for four minutes. RNA was then extracted from the homogenate using the QiaCube equipped with the RNeasy Plus Mini Kit.

cDNA

The RNA concentration was measured using a Nanodrop 2000c (Thermo Scientific) blanked with pH 8.0 Tris-EDTA (Fisher, Hampton, New Hampshire). The stock RNA solution was diluted 5x with Tris-EDTA before sampling for concentration. After determining concentrations, tubes with a total final volume of 14 μL were made for each sample so that there was 1000 ng of RNA in each tube. The remaining volume was

brought to 14 μL with water. The DNA was eliminated and cDNA transcribed according to manufacturer's instructions for the QuantiTect Reverse Transcriptase Kit (Qiagen).

Real Time PCR (RT-PCR) for Neurokinin-1 Receptor (NK1R)

As a preliminary control to assess that gene expression differences are detected using our model and that our RNA extraction method is valid, a housekeeping gene and a gene we believe would change based on previous work were quantified in a mTBI and TT mouse, prior to running the analysis on the microarray chips. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) forward and reverse primers were used as the housekeeping gene, NK1R forward and reverse primers (Table 1) were used as the gene expected to change due to our previous work which saw differences when the NK1R was modulated[8]. The mRNA transcripts were quantified from the cDNA using RT-PCR. Primers were purchased from Integrated DNA Technologies (Coralville, Iowa).

Table 1. Forward and reverse primer sequences of the tested HPRT housekeeping gene and NK1R gene.

Gene	Sequence
HPRT forward	5'-TCG GCT TAC CTC ACT GCT TTC-3'
HPRT reverse	5'-CCT GGT TCA TCA TCG CTA AT-3'
NK1R forward	5'-GAT ACC TCC AGA CCC AGA G-3'
NK1R reverse	5'-GCT GGA GCT TTC TGT CAT G-3'

A 55 μL master mix was created using the QuantiFast SYBR Green PCR Kit (Qiagen) that consisted of 25% SYBR Green, 10% forward primer, 10% reverse primer, and 45% water. 49.5 μL of this master mix was added to each tube (tubes were run in duplicate with a negative of just master mix, and positive control of naïve mesenteric lymph node RNA). 5.5 μL of the cDNA sample (10% of final volume of 55 μL) was added to each tube. 25 μL of the 55 was split into duplicates. HPRT and NK1R were then run according

to manufacturer's instructions on the StepOnePlus RT PCR (Applied Biosystems, Foster City, CA)

Microarray

General procedure, normalization, and quality assessment

The procedure and subsequent analysis were run at the Boston University Microarray and Sequencing Resource Core Facility, funded by CTSA grant UL1-TR001430. The following methods for the microarray procedure are compiled with the help of the BU Microarray Core technicians Adam Gower and Eduard Drizik. All microarray analyses were performed using the R environment for statistical computing (version 2.15.1). Gene microarray analysis was run on a mouse Affymetrix GeneChip® Gene 1.0ST Array System. Mouse Gene 1.0 ST CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average (RMA)[14] in the "affy" R package (version 1.36.1)[15] included in the Bioconductor software suite (version 2.12)[16] and an Entrez Gene-specific probeset mapping (17.0.0) from the Molecular and Behavioral Neuroscience Institute (Brainarray) at the University of Michigan[17, 18]. Array quality was assessed by computing Relative Log Expression (RLE) and Normalized Unscaled Standard Error (NUSE) using the "affyPLM" package (version 1.34.0)[19]. RLE is a measure of the relative quality of each sample (how much the signal was artificially boosted during normalization) compared to other arrays in the batch. NUSE is a measure of the relative agreement between a sample's probes for each gene, compared to the other arrays in the batch. For each sample, median RLE values >

0.1 or NUSE values > 1.05 are considered out of usual limits. All arrays had median RLE and NUSE values within these limits, suggesting samples of similar quality.

Principal Component Analysis (PCA) was performed using the "prcomp" R function with values normalized across all samples to a mean of zero and a standard deviation of one. Differential expression was assessed using the moderated (empirical Bayesian) t test implemented in the "limma" R package (version 3.14.4). Correction for multiple hypothesis testing was accomplished using the Benjamini-Hochberg false discovery rate (FDR)[20]. Human homologs of mouse genes were identified using HomoloGene (version 68)[21].

Positive control gene expression

The expression of several sex-specific genes (Xist, Ddx3y, Eif2s3y, Kdm5d, and Uty) was assessed to estimate the dynamic range of the array, as the female-specific marker Xist and constitutively expressed Y-linked genes serve as strong positive and negative expression controls in female animals, respectively (and vice versa in male animals). In all samples, the expression of Xist was high (~9 $\log_2$ units) and the expression of the Y-linked genes was very low (~2-3 $\log_2$ units), confirming that the mice used were female. This analysis also indicates that the experiment has good dynamic range to identify genes with true differential gene expression.

Statistical analysis

Pairwise t tests were performed for each gene between experimental groups to obtain a t statistic and p value for each gene. A "moderated" t test was used, which is a Bayesian analysis that does not test each gene independently, but rather, leverages

information from all of the genes on the array to increase statistical power over Student's two-sample t test. It was used because it is a helpful statistical analysis when sample sizes are small, as in this study. FDR correction was applied to obtain FDR-corrected p values (q values). The FDR q value was also recomputed after removing genes that were not expressed above the array-wise median value of at least one array. Genes with low overall expression are more strongly affected by random technical variation and more likely to produce false positive results. The value considered significant for the FDR q value (comparable to a $p<0.05$) is <0.25. Q values represent the probability that a given result is a false positive based on the distribution of all p values on the array. This implies that there is less than a 25% chance that the observed pattern between experimental groups is a false positive. Corrected/adjusted p values such as the FDR q are the best measure of significance for a given test when many genes are tested at once. All FDR q values mentioned will be less than <0.25, which is a good measure of significance given the breadth and sheer number of gene expression values which are factored into the FDR q value.

Gene Set Enrichment Analysis (GSEA)

GSEA (version 2.2.1)[22] was used to identify biological terms, pathways and processes that are coordinately up- or down-regulated within each pairwise comparison. The Entrez Gene identifiers of the human homologs of the genes interrogated by the array were ranked according to the moderated t statistic computed between mTBI and TT. Mouse genes with multiple human homologs (or vice versa) were removed prior to ranking, so the ranked list represents only those human genes that match one mouse gene.

This ranked list was then used to perform pre-ranked GSEA analyses (default parameters with random seed 1234) using the Entrez Gene versions of the Hallmark, Biocarta, KEGG, Reactome, Gene Ontology (GO), and transcription factor and microRNA motif gene sets obtained from the Molecular Signatures Database (MSigDB), version 5.0[23].

RESULTS

Microarray analysis - Principal Component Analysis (PCA)

To analyze broad overall genetic differences between mTBI (n=3) and TT (n=3) lungs at 48 hours, the results of the microarray were analyzed via PCA. PCA is a mathematic algorithm which groups the genetic differences between samples across all 21,187 probesets on the chip into smaller Principal Components (PC) so that larger differences can easily be viewed. The PC1 axis accounts for the most genetic variation. Unfortunately, the groups do not separate on this axis, suggesting that the largest differences between the groups are not due to the treatment. However, the groups revealed strong separation along the PC2 axis which suggests that 24% of all variance in the experiment is due to the biological consequences of mTBI versus TT on the lungs (Figure 2).

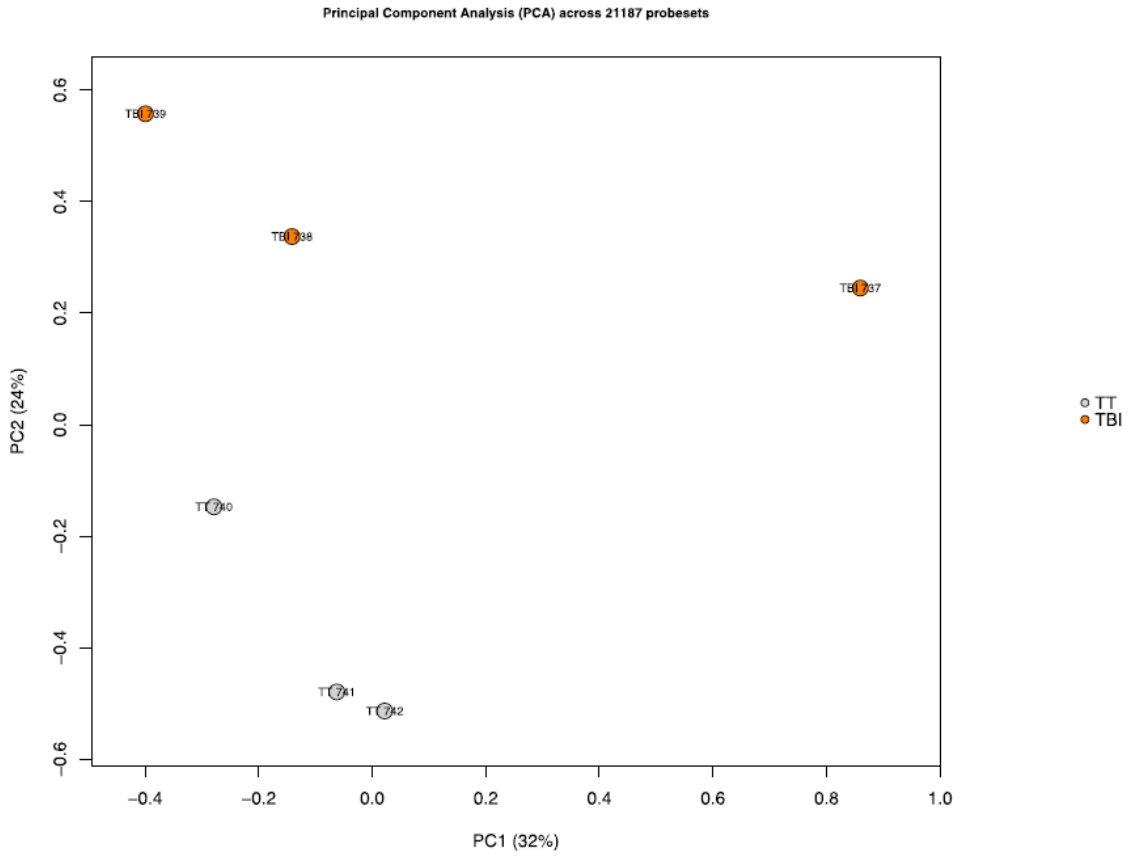

Figure 2. Plot of PC1 vs. PC2, computed across all genes. The PCA plot represents the broad differences in genetic expression of mTBI and TT RNA extracted from mouse lungs. mTBI n=3, TT n=3.

However, further analysis of TBI 737, the sample which separates strongly from both groups, revealed that a piece of thymus or mediastinal lymph node could have been included with the lung as it was collected. We believe this to be the case as TBI 737 had abnormally high genetic expression of B cell and antibody related genes as compared to the other 5 mice (Figure 3).

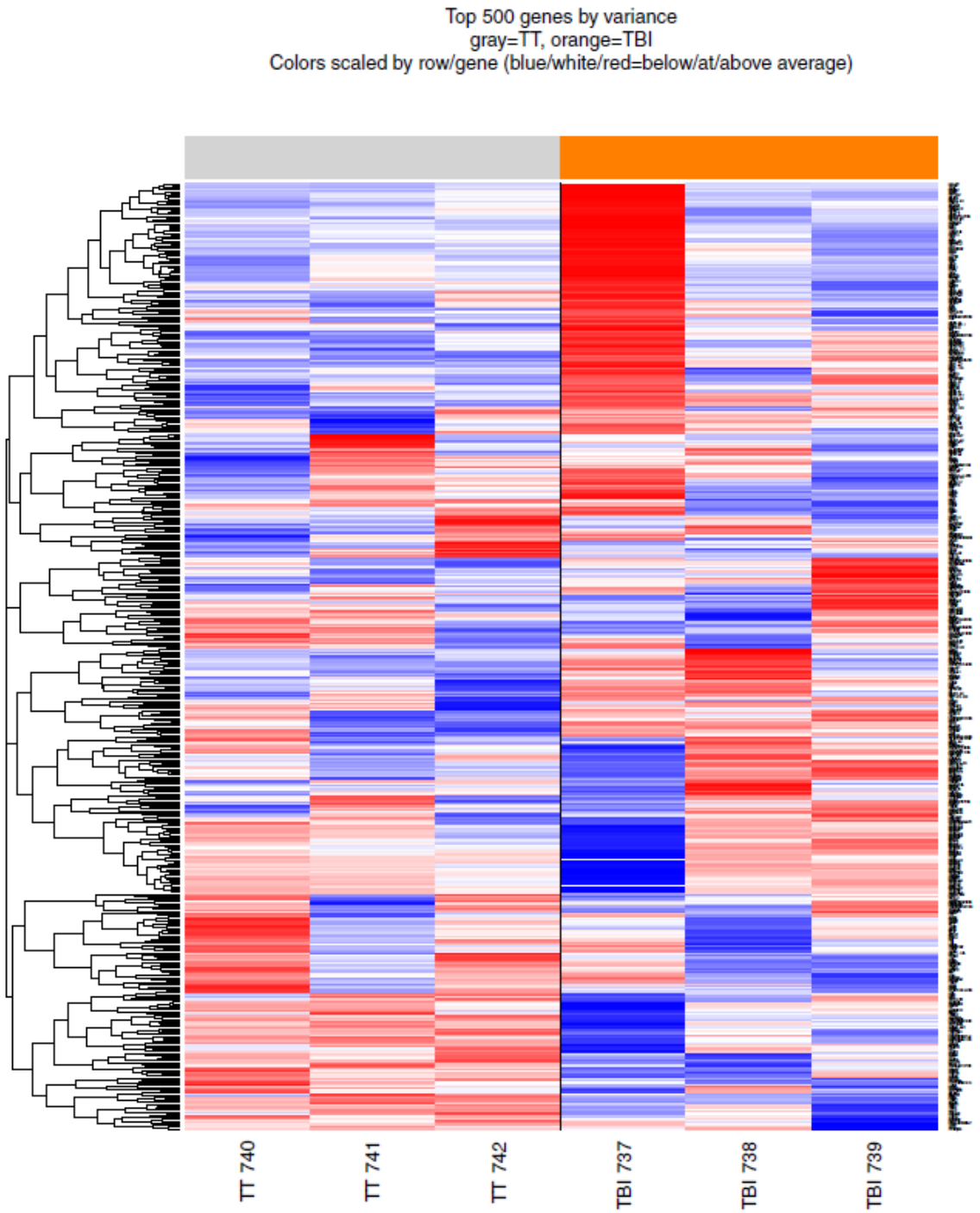

Figure 3. Heatmap of the top 500 genes with largest variance across all samples. TBI 737 has a large portion of genes at the top of the column heavily upregulated compared to all other samples. This is likely due to lymph node contamination as the genes within this section are all canonical B cell genes.

The topmost part of TBI 737 column, which is the deepest red compared to all other mice in that particular gene set, shows that it is far more upregulated than other samples. The genes that are heavily upregulated in TBI 737 support the possible lymphatic tissue contamination as the genes within this deep red section are mostly canonical lymphocytic markers (Bcl11a, Blk, Btla, CD19, CD72, CD79a, CD79b, Ly6d, Ptpn22, Sell). Also, none of the other 5 mice showed any level of genetic upregulation close to TBI 737 in this set of genes, supporting our idea that the sample was contaminated. This lymphocytic contamination could explain the strong separation of TBI 737 along the PC1 axis. For this reason, we felt confident in excluding TBI 737 from the analysis as we were interested in changes in the lung only.

Due to the tissue contamination and subsequent exclusion, we reran all analyses with TBI 737 excluded for a total of three TT and two mTBI mice (Figure 4).

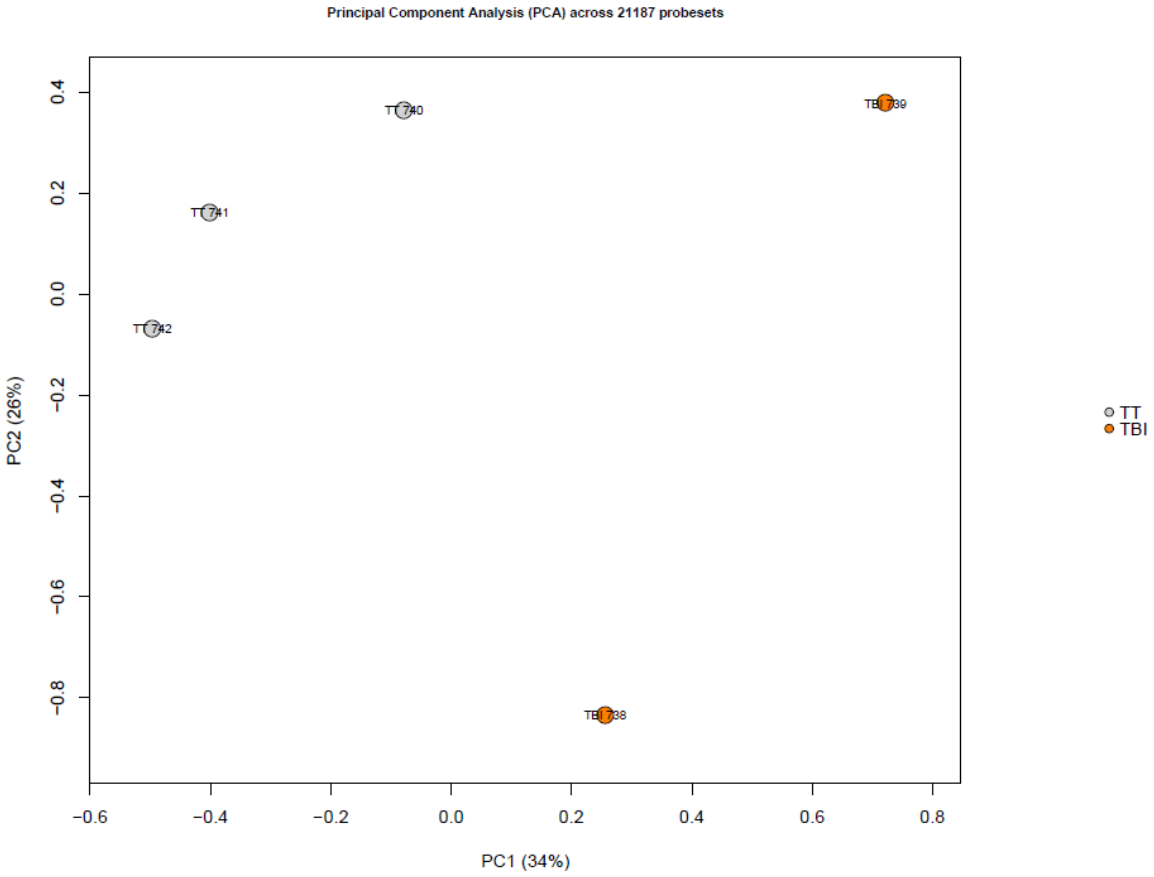

Figure 4. Plot of PC1 vs. PC2, computed across all genes with TBI 737 removed due to contamination. Treatment groups now separate strongly with 34% variance on the PC1 axis, the axis which accounts for the most variation. mTBI n=2, TT n=3.

When the data was rerun without the outlier skewing all the analysis, the PCA plot was rearranged so that the treatment groups still separated, but now on the PC1 axis, suggesting that 34% of the biological differences seen between mTBI and TT were due to the treatment. While the separation is not as visually striking as the original PCA plot, this is likely due to normal biological variability, and the new separation on the PC1 between the groups is encouraging as the PC1 axis accounts for the most variation. From this point onward, all analyses and discussions will be with the TBI 737 outlier removed.

Microarray analysis- Gene Set Enrichment Analysis (GSEA) – Upregulated gene sets in mTBI

GSEA groups sets of genes which are coordinately up or down regulated with respect to a given comparison. The gene sets are related to biological pathways or processes and the genes included in my analysis are compared to publicly available gene sets at the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). Within this database are gene sets which represent well-studied pathways and other gene sets generated by the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), Biocarta, and Reactome. The gene sets created by these four entities correspond to biological functions, locations, or pathways. A gene set that is significantly different between mTBI and TT with a $p<0.05$ and FDR $q<0.25$ suggests that the pathway or process could be changed as a result of the mTBI. Of the 433 total gene sets which had a $p<0.05$ and FDR $q<0.25$, 98 were upregulated in mTBI compared to TT lungs.

ATP synthesis

Of the 94 upregulated gene sets, 20 are related to ATP synthesis or mitochondrial structure and function (Table 2). The most significantly upregulated gene sets are related to oxidative phosphorylation, an example of which is seen in Figure 5.

Table 2. 20 ATP synthesis or mitochondria related gene sets which are upregulated in mTBI lungs vs. TT with a FDR q<0.25. GSEA, Gene Set Enrichment analysis; NES, normalized enrichment score; *P* value <0.01; FDR (false discovery rate) Q <0.25

GSEA Set Name	NES	Nominal P Value	FDR Q Value
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_ SYNTHESIS _BY_CHEMIOSMOTIC_COUPLING_ AND_HEAT_PRODUCTION_BY_UNCOUPLING_PROTEINS_	2.86	0.000	0.0000
KEGG_OXIDATIVE_PHOSPHORYLATION	2.68	0.0000	0.0000
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT	2.84	0.0000	0.0077
REACTOME_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_ TRANSPORT	2.30	0.0000	0.0011
MONOVALENT_INORGANIC_CATION_TRANSMEMBRANE_T RANSPORTER_ACTIVITY	1.80	0.0062	0.0831
MITOCHONDRIAL_RIBOSOME	2.28	0.0000	0.0013
MITOCHONDRIAL_PART	2.20	0.0000	0.0029
MITOCHONDRIAL_MEMBRANE_PART	2.20	0.0000	0.0030
MITOCHONDRIAL_ENVELOPE	2.03	0.0000	0.0168
MITOCHONDRIAL_LUMEN	1.94	0.0000	0.0363
MITOCHONDRIAL_MATRIX	1.92	0.0020	0.0 420
MITOCHONDRIAL_MEMBRANE	1.87	0.0000	0.0561
MITOCHONDRIAL_OUTER_MEMBRANE	1.84	0.0102	0.0705
CELLULAR_RESPIRATION	1.84	0.0083	0.0683
MITOCHONDRION	1.82	0.0000	0.0749
REACTOME_MITOCHONDRIAL_PROTEIN_IMPORT	1.77	0.0061	0.0970
MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS	1.67	0.0143	0.1532
ELECTRON_CARRIER_ACTIVITY	1.57	0.0067	0.2237
MITOCHONDRIAL_RESPIRATORY_CHAIN	1.55	0.0455	0.2355
MITOCHONDRIAL_TRANSPORT	1.55	0.0342	0.2382
MITOCHONDRIAL_INNER_MEMBRANE	1.54	0.0258	0.2463

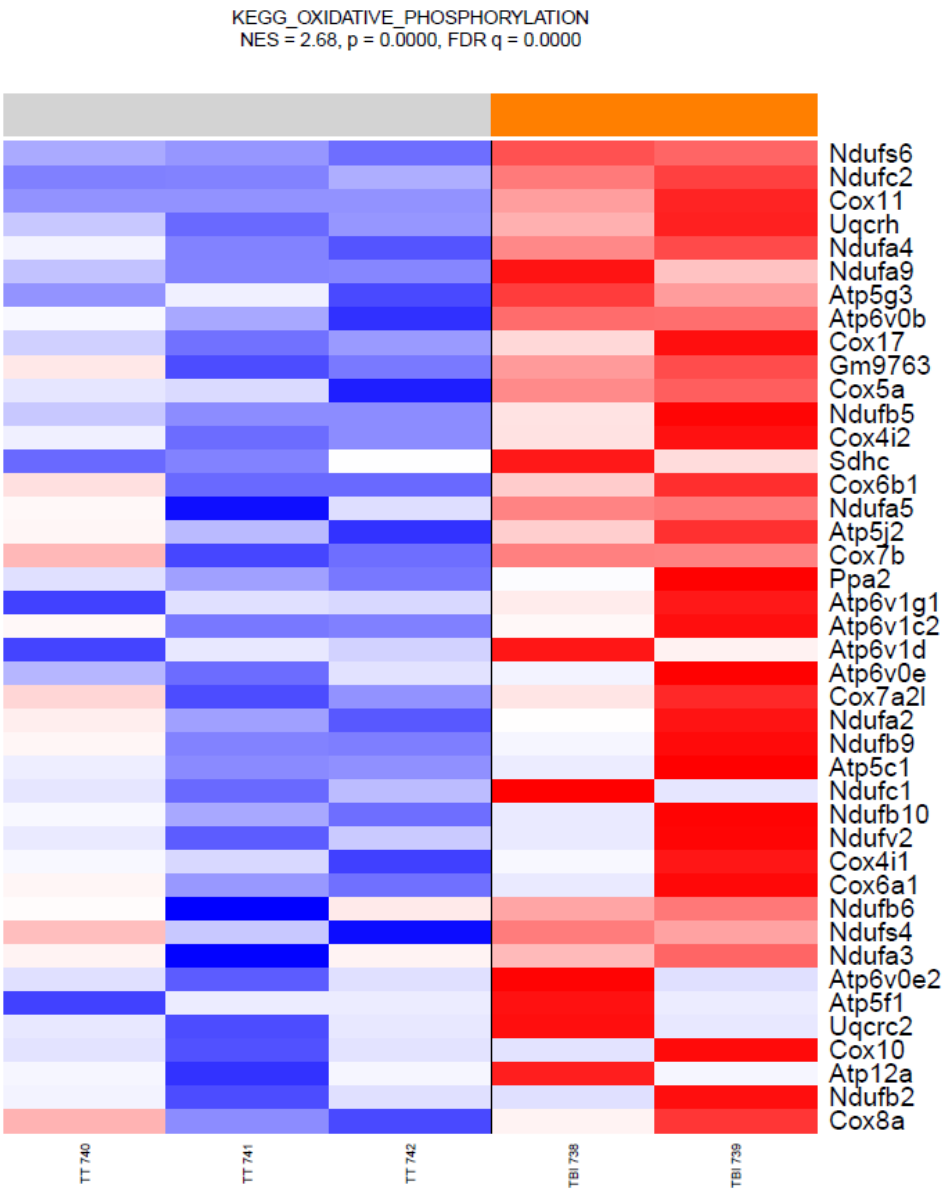

Figure 5. Example of leading edge gene heatmap of the genes upregulated in mTBI (orange) vs. TT (gray) within the KEGG classified "Oxidative Phosphorylation" gene set. These are the leading edge genes within the set, meaning they contribute the most to the statistical significance of this set's upregulation in mTBI (red and blue = upregulated and downregulated compared to mean expression, respectively).

Other upregulated sets

Our mTBI model provides an increased survival which we believe is partly the result of a neuro-immune response stemming from the brain injury. Our work has focused

on the neuropeptide Substance P (SP) however; there are broad effects of a brain injury which likely affect the immune response. In support of this, the "Neurotransmitter Receptor Activity" pathway is significantly upregulated with a FDR $q<0.25$ (0.196), suggesting that the receptors for messages originating from the brain are receiving instructions to upregulate in mTBI versus TT lungs. This could mean that the lungs of mTBI mice have more active neurotransmitter activity, so that they could be more receptive and responsive to any signals coming from the brain, like SP, which we believe is a central component of our increased survival.

Of interest in our model's enhanced immune response to a *Psd* infection, "Biocarta Cytokine Pathway" and "Reactome Downstream Signaling Events Of B Cell Receptor BCR" are upregulated in mTBI with a FDR $q<0.25$ (0.187, 0.197 respectively). While this particular microarray analysis is limited to measuring messages sent in the form of mRNA and not levels of actual protein, the "Reactome Activation of the mRNA Upon Binding of the Cap Binding Complex and EIFS and Subsequent Binding to 43S" and "Translation" pathways are all upregulated in mTBI versus TT with an FDR $q<0.25$. This indicates that the pathways involved in turning mRNA into protein could be more active in mTBI lungs than TT, suggesting the possibility that in mTBI lungs more so than TT lungs, these messages could be turned into protein (Figure 6).

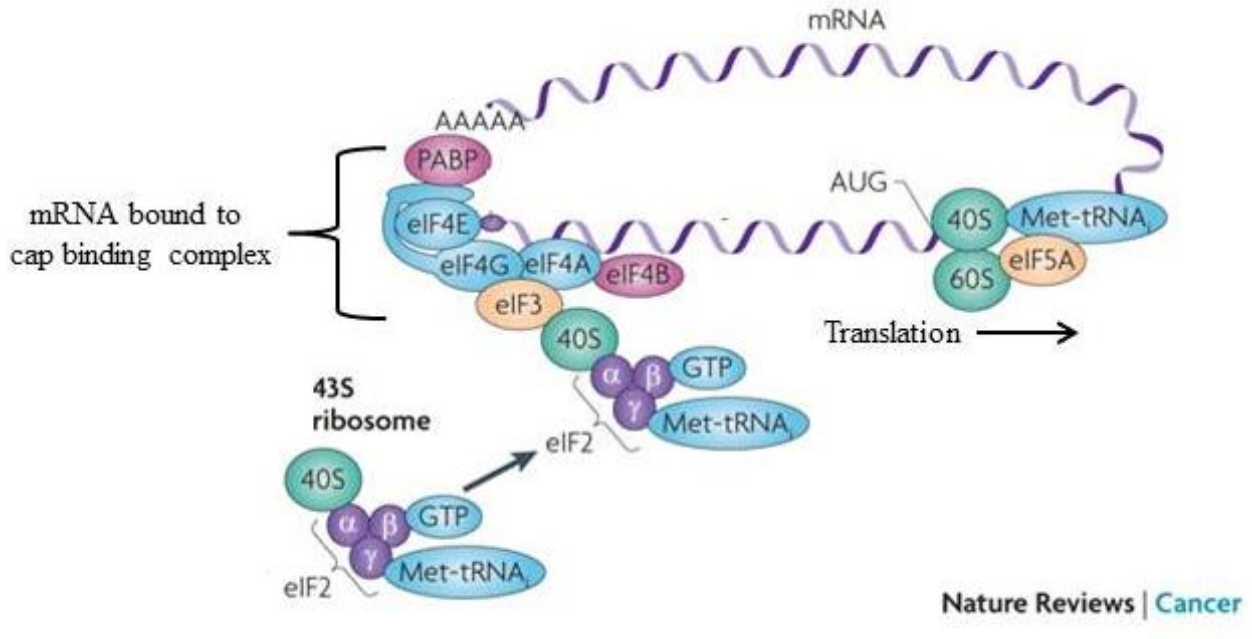

Figure 6. Schematic representation of the mechanism likely to be more active based on upregulation of the gene sets "Reactome Activation of the mRNA Upon Binding of the Cap Binding Complex and EIFS and Subsequent Binding to 43S" and "Translation" in mTBI vs. TT lungs. Upregulation of these pathways imply that translation is more likely in the mTBI lungs as the pathways are integral steps to the production of protein. First, the mRNA binds to the cap binding complex (made of various eukaryotic initiation factors indicated by the bracket), then joins with the 43s pre-initiation complex to make the active 80s ribosome (40s/60s). After formation of the 80s ribosome, translation takes place, turning the mRNA into protein. The genes included in the pathways making this mechanism imply that mTBI is more likely to produce protein than TT lungs because the genes constituting these mechanisms are upregulated mTBI mice vs. TT (adapted from Silvera et al., 2010[24]).

Microarray analysis- Gene Set Enrichment Analysis (GSEA) – Downregulated gene sets in mTBI

Integrin, extracellular matrix, adhesion molecules

In mTBI lungs, there are multiple downregulated gene sets related to inter-cell, intra-cell, and extracellular connections and organization (Table 3). Our published data of increased neutrophil counts in the lungs of mTBI versus TT lungs after pathogen

challenge is supported by these downregulated gene sets. This genetic change supports the idea that the mTBI lungs immediately prior to infection are "leakier" than TT lungs, possibly allowing for easier extravasation of the recruited immune responders from circulation into the air space. We believe this less impeded entry into the airspace upon pathogen challenge, on account of the leakier lungs, is another factor supporting the increased survival of mTBI mice compared to TT.

Table 3. Gene sets related to inter-cell, intra-cell, and extracellular connections and organization downregulated in mTBI vs. TT lungs with a FDR q<0.25. NES, normalized enrichment score; *P* value <0.01; FDR (false discovery rate) Q <0.25

GSEA Set Name	NES	Nominal P Value	FDR Q Value
KEGG_CELL_ADHESION_MOLECULES_CAMS	-1.9	0.000	0.0490
CELL_SUBSTRATE_ADHESION	-1.56	0.036	0.1760
CELL_MATRIX_ADHESION	-1.58	0.020	0.1234
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	-1.8	0.000	0.0763
KEGG_ADHERENS_JUNCTION	-1.71	0.000	0.0676
ACTIN_BINDING	-1.71	0.004	0.0682
REACTOME_CELL_JUNCTION_ORGANIZATION	-1.73	0.002	0.0606
CELL_SURFACE	-1.74	0.000	0.0540
REACTOME_ADHERENS_JUNCTIONS_INTERACTIONS	-1.88	0.002	0.0204
ACTIN_CYTOSKELETON	-1.93	0.000	0.0151
INTEGRIN_BINDING	-1.96	0.000	0.0120
EXTRACELLULAR_REGION	-2.02	0.000	0.0065
INTEGRIN_COMPLEX	-2.08	0.000	0.0033
BASEMENT_MEMBRANE	-2.41	0.000	0.0002
STRUCTURAL_CONSTITUENT_OF_CYTOSKELETON	-1.67	0.010	0.1211
COLLAGEN	-2.1	0.002	0.0152
KEGG_FOCAL_ADHESION	-2.36	0.000	0.0000
REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS	-2.7	0.000	0.0000
REACTOME_COLLAGEN_FORMATION	-2.94	0.000	0.0000
REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION	-2.83	0.000	0.0000
KEGG_ECM_RECEPTOR_INTERACTION	-2.70	0.000	0.0000

Glycosaminoglycans (GAG)

GAGs are known to aid in cell migration and *Psd* pathogenesis by facilitating attachment to the host, amongst other functions[25,26]. mTBI mice had downregulated GAG

metabolism pathways, which could make their lungs a more difficult environment for *Psd* to establish an infection. Heparin, a GAG, is thought to *inhibit* cell migration of leukocytes[27], with heparin metabolism pathways downregulated in mTBI lungs prior to infection; the leukocytes in an mTBI lung could migrate more easily upon infection. Dermatan sulfate, another GAG, has been shown to be released from host tissues following *Psd* infection and *inhibit* the antibacterial functioning of neutrophil released factors[28]. With this pathway downregulated, the initial immune response of mTBI leukocytes could be more effective than TT. This is because at the time of infection, it is likely there is less of the inhibitory dermatan sulfate present in mTBI lungs. With less inhibitory dermatan sulfate in the mTBI lungs as suggested by the downregulated pathway, the mTBI lungs could be an environment where leukocytes operate more effectively.

Psd virulence related

Gene sets indirectly involved in *Psd* virulence were downregulated in mTBI lungs. *Psd* causes tissue damage via exotoxin A which enters cells via receptor-mediated endocytosis[29,30], a pathway that is downregulated in mTBI lungs (Figure 7).

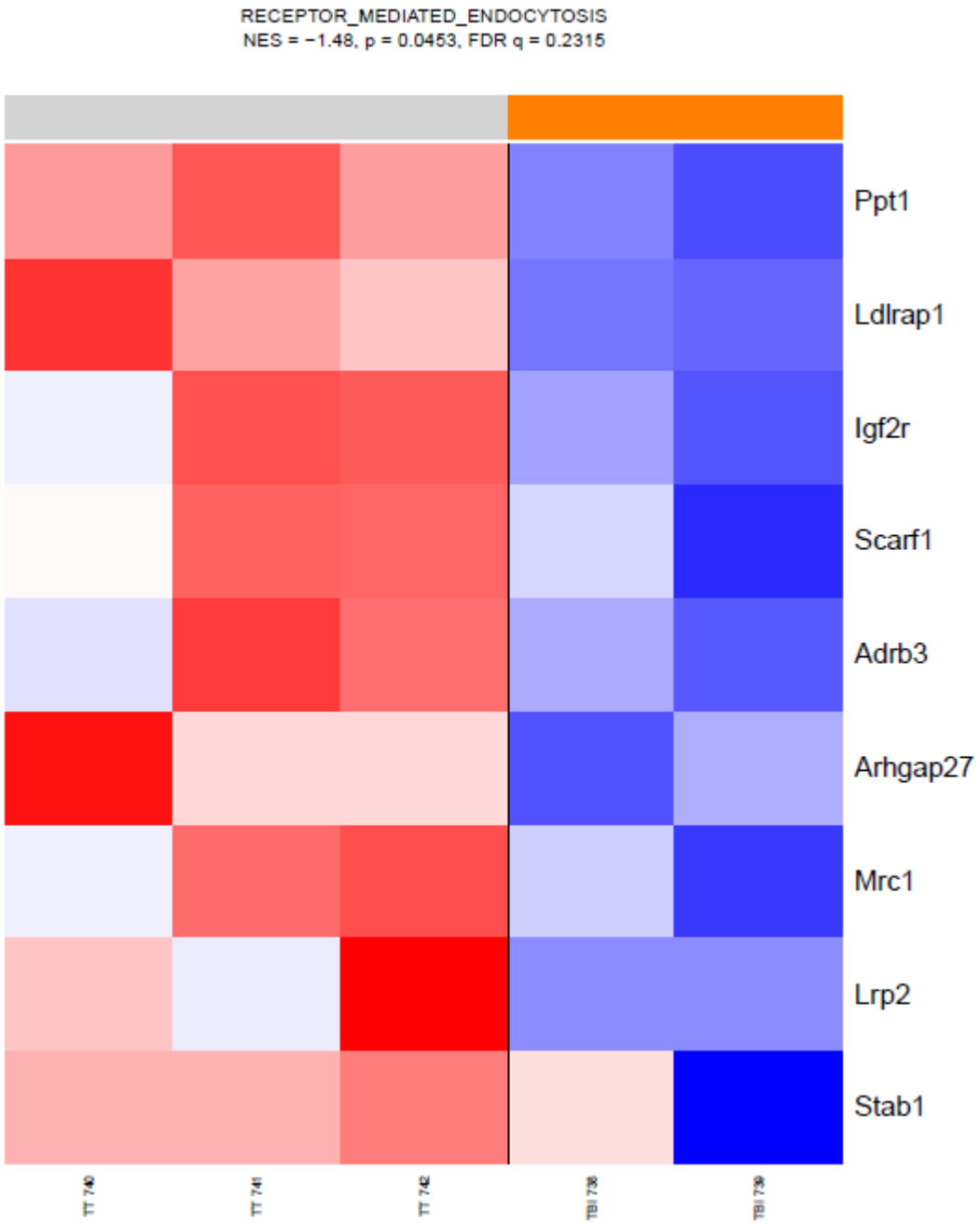

Figure 7. Heatmap of the genes downregulated in mTBI (orange) vs. TT (gray) within the "Receptor Mediated Endocytosis" gene set. Receptor mediated endocytosis is a proposed mechanism of entry for *Psd*'s virulent exotoxin A into host cells. Downregulation of this pathway could inhibit one mechanism of damage *Psd* utilizes, possibly providing protection to mTBI lungs. (red and blue = upregulated and downregulated compared to mean expression, respectively)

It is believed that *Psd* produces extracellular phospholipase C (PLC) that is secreted through its inner membrane and is thought to be another contributing factor to its virulence[31,32]. In mTBI lungs, the host-derived components which manifest the effects triggered by exogenous PLC are downregulated in mTBI lungs ("Reactome PLC Mediated Cascade, NES = −1.60, p = 0.0384, FDR q = 0.1530). This could be beneficial to the mTBI mice because the PLC produced by the pathogen, which is usually detrimental in the host, could be less effective as the downstream pathway triggered by the pathogen-produced PLC is downregulated in mTBI lungs.

Also of interest is the downregulation of pathways related to proteins that *Psd* could use to adhere to the epithelium to initiate infection, like laminin (Figure 8). With fewer messages being sent to produce these proteins, the *Psd* could have a more difficult time initiating an infection, possibly contributing to the increased survival of the mTBI mice.

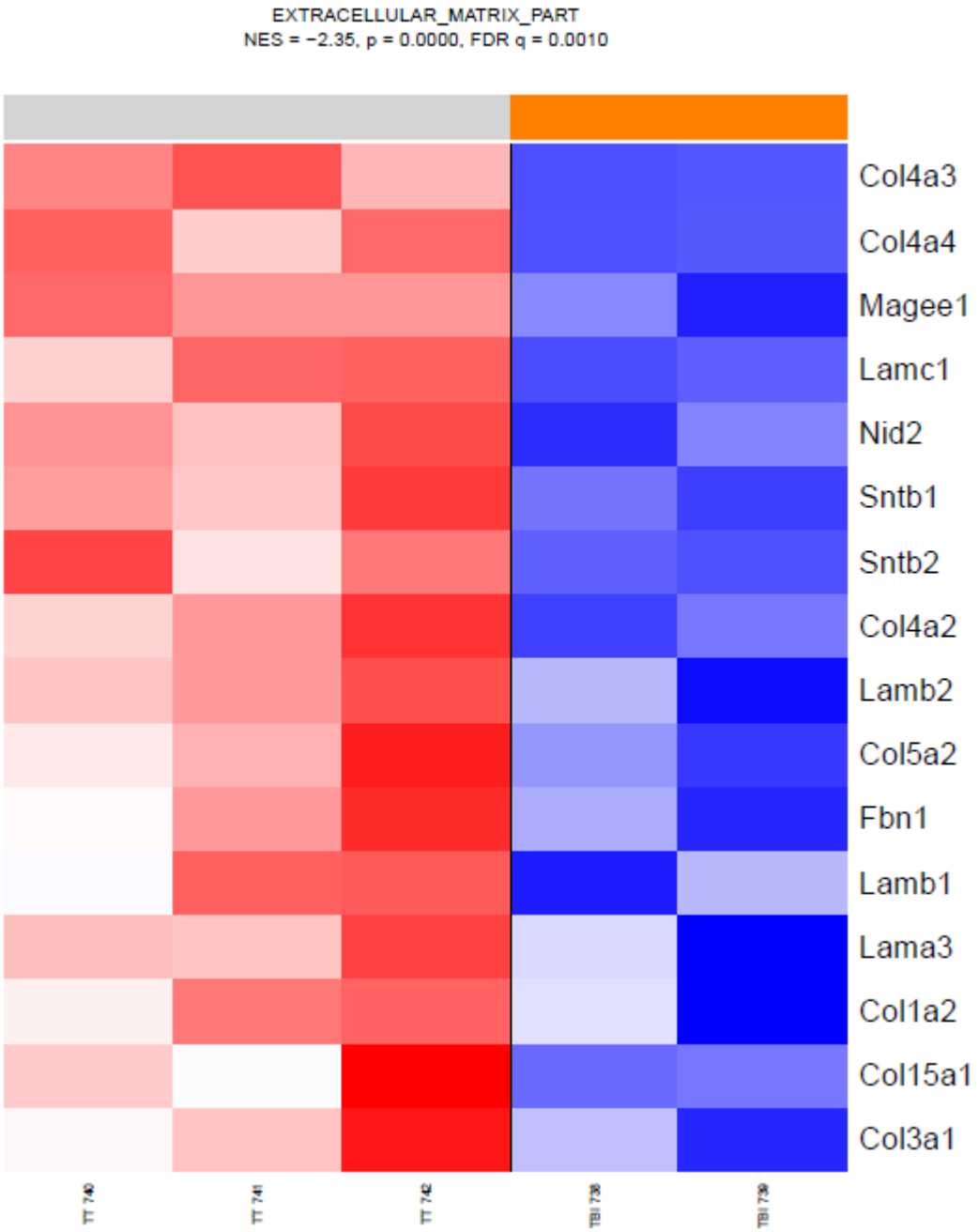

Figure 8. Heatmap of the genes downregulated in mTBI (orange) vs. TT (gray) within the "Extracellular Matrix Part" gene set. Lamc1, Lamb1, Lamb2, Lama3 are all genes related to the basement membrane protein Laminin, which *Psd* is thought to use as an attachment point to initiate infections. (red and blue = upregulated and downregulated compared to mean expression, respectively)

DISCUSSION

A large portion of gene sets that were upregulated in mTBI lungs were pathways related to ATP synthesis or mitochondrial structure and organization. These upregulated gene sets imply the enhanced production of ATP which could have multiple beneficial effects on the innate immune system once challenged with *Psd*. If the mTBI lungs prior to infection are more bio-energetically prepared than TT lungs at 48 hours, once the pathogenic insult occurs, it is likely that the mTBI lungs can more effectively create a chemotactic response via cytokine production. It is thought that there are low levels of extracellular ATP existing in a "halo" around normal resting cells[33]. Based on the genetic expression of mTBI lungs, it is reasonable to believe that the resting cells in mTBI lungs could have more extracellular ATP existing in this "halo". This low level of normal extracellular ATP, higher in the mTBI lungs, can have a beneficial effect on the immune system. This is because low levels of extracellular ATP have a chemotactic effect on neutrophils, monocytes, macrophages, and immature dendritic cells[34], implying that there could be more chemotaxis to mTBI lungs by immune responders once an influx of chemokines is released after infection, allowing mTBI to combat the infection more effectively, accounting for increased survival.

Additionally, increased oxidative phosphorylation is thought to raise the amount of reactive oxygen species (ROS) available to the immune system[35]. ROS production is crucial for the ability of neutrophils and macrophages to phagocytose and eradicate bacteria effectively[36,37]. With more genetic expression suggesting an increase in electron movement through oxidative phosphorylation in the lungs of mTBI mice, it is possible

mTBI lungs have more production of ROS in their eukaryotic cells[35]. Given the method for RNA extraction in this experiment, it is unclear where this possible increase in ROS is stemming from. Since entire sections of lung were removed and homogenized, this increase in ROS production could be either in the epithelial cells of the lung or within immune cells which happened to be included in the lung digest. If the possible increased ROS was sourced from immune cells trapped in the digest, this suggests that the mTBI neutrophils are better equipped to eradicate an infection. If the ROS production is in the epithelial cells of the lung, it is unclear if these cells could transfer ROS to the immune responders and thus provide them with an enhanced killing ability.

Innate immune system responders can be triggered by damage-associated molecular patterns (DAMPs). DAMPs are endogenous cellular components that are released from damaged or necrotic cells after tissue damage resulting from non-pathogenic or pathogenic sources[38]. DAMPs serve as a warning signal to the organism that a dangerous situation has developed which is destroying the cell and thus, they recruit immune cells to clear the damage or pathogen. DAMPs are derived from the nucleus, cytoplasm, exosomes, extracellular matrix, and plasma[38]. There are also non-protein DAMPs such as uric acid, RNA, DNA, and ATP[39]. With genetic expression of ATP production pathways being significantly upregulated compared to TT lungs, it is possible that the mTBI lungs are filled with more ATP at 48 hours. So, upon infection with *Psd* at the 48 hour time point, the cellular damage caused by the pathogen could result in a far larger release of DAMPs (i.e. ATP) in the mTBI lungs.

ATP, now extracellular and acting as a DAMP due to the damage from pneumonia, has a number of augmenting effects on the innate immune system. Extracellular ATP acts as a chemotactic agent for neutrophils, as seen when injected intraperitoneally into mice[40]. In addition to the neutrophils recruited solely by ATP in these mice, the levels of MIP-2 and KC, two mouse chemokines, were also shown to increase. This is supported by a separate study showing that extracellular ATP triggers macrophages to release MIP-2 and increase migration of neutrophils to the site of ATP[40]. Thus, two chemotactic molecules are generated in addition to the chemotactic extracellular ATP, vastly increasing the potential number of recruited neutrophils to the site of ATP release. Upon lung damage after *Psd,* it is likely the increased neutrophil recruitment we see in mTBI lungs could be in part due to higher levels of the chemotactic extracellular ATP.

While migration of neutrophils to the site of damage is increased by extracellular ATP, once the neutrophils have arrived, the larger amounts of extracellular ATP in mTBI lungs could increase their adhesion to the endothelial cells, an effect supported by numerous sources[41, 42,43,44,45,46,47]. Specifically, the increased levels of extracellular ATP likely to be found in mTBI lungs have been shown to act through the $P2X_7$ receptor to activate (NF)-κB, which upregulates E-selectin, a protein necessary to initiate the rolling of neutrophils[48,49], a crucial step involved in extravasation.

Extracellular ATP not only increases the number of neutrophils at the site of its release, it is suggested to increase the effectiveness of the neutrophil's bacterial clearance ability by increasing the production of superoxide, a component of ROS[50]. Our gene set

data supports the hypothesis that the cells in mTBI lungs at 48 hours have greater levels of ATP which could provide more energy to the lung for a more robust immune response than TT lungs once they receive pneumonia. However, once the pneumonia has started damaging cells, the data supports the hypothesis that mTBI lungs will release far more ATP, due to the upregulation of ATP synthesis gene sets, which can then act as a chemotactic and immune augmenting DAMP. With more extracellular ATP in mTBI lungs, there could be enhanced recruitment, better extravasation into the lung space, and increased killing ability of neutrophils to fight the infection, leading to a more effective immune response and our observed increased survival of mTBI mice. This hypothesized mechanism of action is supported by our previous work which shows increased numbers of neutrophils and decreased bacterial colony forming units (CFUs) in the bronchoalveolar lavage of mTBI lungs 4 hours after pneumonia insult[7].

Interestingly, the majority of gene sets were downregulated in mTBI lungs and most functions involved inter-cell, intra-cell, and extracellular connections and organization. Epithelial cells of mTBI lungs could have an altered organization, structure, and connection compared to TT, possibly providing an environment for quicker extravasation of circulating leukocytes to the airspace following *Psd* infection.

Psd infections begin with adhesion to the endothelial cells in the lung[51]. While many of these connections are still unknown, there is some literature support for *Psd* utilizing cellular components which are downregulated in the mTBI lungs according to GSEA. In particular, *Psd* has been suggested to adhere to extracellular matrix proteins of the epithelium, like laminin, which constitute the basement membrane of epithelial cells

and are revealed upon cellular damage[52]. Many of the genes for laminin are downregulated in the "Extracellular Matrix Part" gene set seen in Figure 7. Given that the gene set is downregulated in mTBI lungs, it is possible there are fewer of these proteins in the epithelial cells. So, after they are damaged by the infection, in mTBI lungs the cells could have fewer proteins like laminin, leading to a decreased ability of *Psd* to adhere to lungs. This could interfere with their mechanism of infection, making them less virulent and easier to phagocytose than the *Psd* which could securely attach to the extracellular matrix proteins in TT lungs. Glycosaminoglycans are polysaccharides consisting of disaccharides made of amino and uronic sugars[53]. One amino sugar which makes up glycosaminoglycans is N-Acetylglucosamine, which has been suggested as a molecule which *Psd* can attach to, allowing the initiation of infection[54]. Supporting the increased survival of mTBI mice due to a smaller bacterial burden, mTBI lungs had multiple glycosaminoglycan pathways downregulated, suggesting the expression of fewer attachment points for *Psd* in these mice.

The combination of increased upregulation of ATP and mitochondrial gene sets as well as downregulated *Psd* virulence related sets in mTBI lungs could lead to an environment better suited to fighting an infection than blunt trauma. While the cause of these genetic changes is unknown, it is likely they are based off the downstream effects of the mTBI and subsequent release of SP. A limitation of the study is the RNA extracted and measured are just instructions for the cells to produce proteins. Whether or not the instructions are carried out, and the desired protein is actually manufactured, is unknown. However, the differences in RNA expression of these genes are significant and therefore

it is likely that even if all of the RNA is not being translated, in mTBI, these messages are becoming translated into protein more frequently than TT. This assumption can be made given that in our previous experiments, we have shown that there is an increase in survival of mTBI mice when challenged with pneumonia[7,8].

Future studies will examine the more tangible aspects of the upregulated gene sets. To determine if the message for ATP and mitochondria is being translated into a real result, ATP will be measured in the mTBI lungs 48 hours after trauma using one of the many available commercial assays. To determine if the downregulated gene sets are being reflected beyond just the genetic message, lung leakage 48 hours after trauma will be assessed in mTBI lungs with two protein assays to assess lung leakage. The first procedure will be a Bradford assay of the BAL at 48 hours to measure large molecular leakage into the airspace. The second will be intravenous administration of Evans Blue via the tail vein prior to mTBI followed by sacrifice and BAL 4, 24, and 48 hours afterwards to assess the amount of albumin (which Evans Blue binds to) that has passed from circulation into the lung space. Histology will also be performed and assessed to determine if there is any identifiable separation in inter- and intra-cellular junctions in mTBI lungs. We would hope to see higher levels of protein and albumin in the BAL of mTBI mice and large inter-cellular junctions to support the hypothesis that the mTBI lungs are "leakier" and thus allow neutrophils easier entry into the airspace once infected with pneumonia, contributing to their increased survival.

While many experiments need to be done to confirm the findings, based off the significance of the statistical analysis of the microarray data, it is likely that the

environment in which the mTBI mice mount their immune response is more energetically prepared and tailored for a successful eradication of the pathogen and thus is likely a contributing factor to our increased survival findings.

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CURRICULUM VITAE