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Gene Expression Analysis of Age and Sex Dependent Effects in the Brain by Acute Inflammatory Response, Lipopolysaccharide

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by

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I want to thank all members in the Carson Lab for their endless support and Dr. Carson for the continuous mentorship.

Dedication

Thank you to my incredible family my Mom, Dad, Sister Brianna and Brother Aiden. Thank you, Robert, for always providing unconditional love and support. And my sweet dog Charlie.

ABSTRACT OF THE THESIS

Gene Expression Analysis of Age and Sex Dependent Effects in the Brain by Acute Inflammatory Response, Lipopolysaccharide

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University of California, Riverside, December 2021
Dr. Monica J. Carson, Chairperson

Transient receptor expressed on myeloid cells 2 (Trem2) plays a role in the Central Nervous System (CNS) however there is limited understanding of what its role is and how its effects can impact the brain. Trem2 has been seen to be highly expressive on microglia cells, one of the resident immune cells in the brain. Interestingly in the literature Trem2 has been suggestive to play a role in protection during neurodegenerative disease pathogenesis and closely linked to Alzheimer's Disease.

To better understand the role Trem2 may have in the CNS, we have designed experiments to study how the depletion of Trem2 expression impacts the baseline in our mouse models and further how it may impact the immune response when challenged by a

proinflammatory stimulant known as lipopolysaccharide (LPS). With the use of mouse model Trem2 knock-out (Trem2KO) in comparison to Wild-Type (WT), data was collected to be able to understand the implications the deletion has on CNS- related pathways. In conjunction with comparison of the two genotypes, studying the differences between the two sexes of male and female is an important factor that experimental studies have lacked to demonstrate. In addition to sex differences our experiments focused on studying differences across age groups; specifically looking at 3 ages including postnatal 15 days (p15), 3 months, and 2 years, equivalating a lifespan. Ultimately, the data was able to show that both age and sex are main drivers in determining the differences further being highly dependent based on the analysis. In basal analysis, data demonstrated sex played the greatest impact in differences whereas in inflammatory response the data suggests age plays the greatest impact.

Table of Contents

List of Figures

Introduction

Neurodegenerative diseases are known to occur when nerve cells that reside in the brain or peripheral nervous system, lose their functional role and worse die [1]. As humans inevitably age the risk for having a neurodegenerative disease increases significantly. The most concerning part of this truth is that while there are short-term relief treatments for some of these diseases, still there is no cure to stop the progression. A neurodegenerative disease known all too well worldwide is Alzheimer's Disease (AD). The Alzheimer's Disease Association presented a 2021 report stating an estimated 6.2 million Americans are living with the disease today [1]. This number can only be expected to increase in the years to come as humans continue to live longer. Many neurodegenerative diseases today, including Alzheimer's Disease, still remain a question as to their origin, mechanism of growth, and how to stop this progression leading to brain. For scientists this is a call for action.

What is known about Alzheimer's Disease is limited and still has much to be understood as our brains and bodies are complex working systems. Alzheimer's Disease is characterized by the buildup of amyloid plaques and presence of neurofibrillary tangles in the brain [2]. Furthermore, neurons lose function and become unable to transmit messages to other parts of the brain, and throughout the body [2]. It has been studied that the disease initially begins in parts of the entorhinal cortex and hippocampus regions of the brain where memory is involved [2]. It is for this reason that impairment in memory is an early symptom and potential indicator of the disease. As the disease progresses it takes over regions in the cerebral cortex, this region of the brain is responsible for social

behaviors, reasoning, and language [2]. While the symptoms become increasingly visible to those around the impacted individual and to the individual themselves as the disease progresses, it is the mechanism that is still a great speculation and where research is still heavily centered to understand. Importantly studies have found that the risk of AD has been found to be 1.5 to 2 times higher in females than males, and risk increases especially in those over eighty years of age [11]. This can be inferred that AD impacts differently based on sex and age, which are two factor our data heavily focuses on as impacting roles.

Microglia and Trem2

Microglia are immune cells that specifically reside in the Central Nervous System (CNS). Recent literature has been suggestive that genetic variants of Trem2, which is a cell-surface receptor that can be seen on myeloid cells, has been found to give higher risk of Alzheimer's Disease. Specifically in the brain, Trem2 has been highly shown to be expressed on microglial cells [3]. The expression of Trem2 has been found highly in white matter, hippocampus, and neocortex which interestingly coincides with regions where pathological features occur in AD patients [9]. Furthermore, suggesting that the innate immune system and cells including microglia can have important roles in AD pathogenesis. Emerging evidence has drawn to the idea that Trem2 is responsible for repressing microglia-mediated cytokine production and secretion which ultimately leads to a suppressed inflammatory response which can be proposed to be reason that can prevent inflammation induced bystander damage to neurons in the brain [9].

With the literature suggesting this potential linkage it has presented an area for further research to understand Trem2, its role in microglia, and how it may have implications to neurodegenerative pathogenesis.

To better understand the potential role Trem2 may play on microglia expression in the CNS, a mouse model with a Trem2 knock-out (Trem2KO) signature will be used and studied to see how data observed in neuropathological pathways compare to their Wild-Type (WT) counterparts. With the deletion of Trem2 it is suggestive that microglia clustering to amyloid-beta plaques would be impaired, cause increased autophagy, impair energetic metabolism, and increase amyloid-beta plaque load in AD mouse models [4]. With the literature suggesting such, it can be inferred that potentially Trem2 could play a role in protection against amyloid-beta plaques and further against neurodegenerative disease progression related to this characteristic. By extension of this hypothesis by depleting the expression of Trem2 on microglia it can be hypothesized that inflammatory responses would worsen and become either non-reactive or hyper reactive to the inflammatory insult.

Lipopolysaccharide

For this research it was imperative that our samples went through an immune inflammatory response that would express a systemic response to ultimately study how this effect impacts the CNS and brain residing genes and cells. LPS is a large glycolipid produced by gram-negative bacteria [5]. Due to the fact that this glycolipid takes up much of the cell surface, it has a role in creating a permeable barrier which protects the

cell from toxic molecules entering the cell [5]. While although LPS serves as a protective barrier to the bacteria it also plays a role as an inflammatory insult to the host immune system when invading the host. Since LPS is on the surface of bacterial pathogens it causes the host immune system to respond to its presence as a pathogen-associated-molecular pattern (PAMP). LPS is detected by toll-like receptor 4, TLR4, and triggers the host immune response [5]. As a result, LPS was a suitable stimulant that could create a systemic inflammatory response that can be used to study the difference in inflammatory response based on genotype and throughout the three ages.

Sex Differences

Our sample size with nine females and nine males (3 per age) per genotype in each the naïve and challenged group allow for opportunity to examine if there are sex differences prevalent in the baseline state and the challenged, throughout all three ages. It was of great importance to our lab to make sure to account for opportunity of a sex factor playing an impacting role in differences within genotype and possible differences in inflammatory response. Emerging studies have begun looking at sex differences within experiments however there is a great gap in what we know about sex playing an impacting factor because it has been studied limitedly. Majority of studies look strictly at the male sex in their mouse models and this provides limited data that is underrepresented. Klein and Flanagan (2016), have reviewed and noted that sex is a biological variable that affect both self and foreign antigen immune responses,

additionally stating that male and female differences in immunological responses are influenced by sex contributing due to physiological and anatomical differences [13].

There is a great importance that comes with studying sex factors simply due to the fact that males and females are genetically and biologically different. Genetically, females have a XX chromosome versus males showing a XY chromosome this could be a cause for potential differences. It can be noted that one of the most prevalent sex differences that are in the brain is the fact that in every neuron, glia, or other cell type carries either the complement of male chromosomes (XY) or female chromosomes (XX) however not both (Arnold and Burgoyne, 2004) [10]. Emerging studies have been linked to better understanding sex chromosome genes that can be attributing to brain sex differences present. Literature has looked closely at the Y-chromosome genes, SRY (Sex-determining Region on the Y chromosome). This gene is passed on from father to son through the Y chromosome, therefore meaning it is not present in females. If the SRY gene is not present then testes are not formed and the female phenotype develops [12].

SRY is a a crucial transcription factor that plays role in male-sex determination. How it is proposed to do this is by directing embryonic biopotential gonads to develop into testes instead of ovaries (Koopman et al., 1990; Sinclair et al., 1990) [12]. The SRY gene has implications to be directing male driven gene expression. More specifically suggestive that this SRY gene in the substantia nigra part of the midbrain in males can control tyrosine hydroxylase (TH)-expressing neuron and is suggestive of a direct male-specific effect on the brain that can only be expressed in males [15]. Due to the potential male specific gene expression, it is suggestive that females must be involved in own

compensatory pathways to equivocate through a female-specific mechanism to result in equal functional output taking place. Ultimately suggestive that there are sex different mechanisms taking place in the CNS [16].

From biological stance females also go through estrous cycles that can involve a fluctuation of hormones and estrogen that males do not, rather their hormones are differently expressed; nonetheless different and can be another potential reason for differences in responses in experiments. Our lab is proud that we have the ability to give representation to both sexes because it in fact will play a role in our data and it is important to highlight such. An important note mentioned in the literature is the possibility that brain diseases that are sexually dimorphic, it can be seen that one sex is protected more than in the other from the disease. It is due to this reason that identifying sex-specific protective agents can play a huge role in better understanding potential mechanisms, therapies, and identifying new targets in study [10].

Effects across Age

Another important factor that we wanted to study in our own data involve the differences age can play. In the sample size utilized for this data a total of 72 mice were involved to ensure representation would be uniform to study three time point ages. These ages include postnatal 15 days (p15), 3 months, and 2 years. The p15 age reflects weaning and puberty, 3 months signify young sexually mature adult, lastly 2 years is representative of an aged mouse. These ages were chosen to reflect a lifespan range to study whether baseline differences and inflammatory responses differ depending on age.

Importantly it will allow us to not only study the baseline and inflammatory effects within each age, it will also provide us the ability to compare impacts across the ages and follow a lifespan in the mice.

Methods

Wild-Type Mouse Model

C57BL/6J mice model was used for experimentation. These mice were purchased from Jackson laboratories and living under specific pathogen free (SPF) vivarium. The Trem2KO used were donated by Marco Colonna. All mice were bred and housed at the University of California animal facility under a 12/12-h light/dark cycle with scheduled lights on at 06:00am [7]. A total of 3 samples in each male and female per genotype, per condition, were used per age of intention to study: p15, 3months, and 2 years. In total 72 samples were used to retrieve the data presented today. In doing so we can ensure an equal representation throughout sex, age, and genotype.

Trem2 Knock-Out Mouse Model

Our lab created the Trem2KO mice. How these mice were created was through the transmembrane and cytoplasmic domains of the receptor, which encoded by exons 3 and 4, allowed to target the gene (pMC1neo) in E14.1 embryonic stem through which then injected into the C57BL/6J blastocytes. Following the removal of the neomycin gene, the mice were then backcrossed until >99% of the loci were of the C57BL/6J strain, this was determined by simple sequence length polymorphism typing. The mice used

were graciously donated by Dr. Marco Colonna [7]. As a result of our lab's efforts, we had the expression of a Trem2 global knock out mouse model that are used in the data of this paper.

Intraperitoneal Injection of Lipopolysaccharide (IP-LPS) Stimulant

To stimulate an inflammatory response both C57BL/6J and Trem2KO mice were administered lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 at a dose of 5mg/kg of body weight into their peritoneal cavity (Millipore-Sigma, cat. L5418). These injections were drawn from a concentration dependent on age; p15 received 0.5mg/mL whereas 3 month and 2-year aged mice were given 1 mg/mL [7]. Due to the manner of this stimulant, inflammation has expected peak between 24-48 hours and by 72 hours it is resolved [8]. With this being said, 24 hours after IP-LPS injections the mice were sacrificed.

RNA Isolation and Quality Check

After euthanasia, brains were dissected. Brains were cut by sagittal orientation allowing for half to be utilized in the experiments for RNA isolation. The half-brains were homogenized in 2.5 mL TRIzol (Millipore-Sigma, cat. R2020) using a mechanical homogenizer (Ultra-Turrax T25). The supernatant next was processed in chloroform (Millipore-Sigma, cat. C2432) then the top layer of nucleic acids was collected and added to isopropanol (Millipore-Sigma, cat. I9516) to allow the RNA to precipitate. Next, the isopropanol was removed and the pellet was washed with 80% ethanol a total of three

times. Following this, the RNA was reconstituted with molecular grade distilled water (Millipore-Sigma, cat. W4502) in doing so gives the ability to acquire an RNA concentration around 200-500 ng/μL. To ensure quality and quantity, RNA was checked by Nanodrop 2000 and inspected using an Agilent RNA 6000 Nano Kit (CAT : PN 5067-1511) on an Agilent 2100 Bioanalyzer. Notably samples with RNA integrity number less than 7 were excluded from our analysis [7-8].

Nanostring Advanced Analysis

The RNA isolated was hybridized with Nanostring's Neuropath probe set panel, which encompasses a total of 770 genes. The RNA samples were loaded into the Nanostring nCounter. Ultimately analyzing the data with the nSolver analysis software (version 4.0.70) [7]. How nSolver analysis software organizes the data is by summarizing the genes associated to each pathway and finding its single score, known as its pathway z-score. Within a heatmap, these pathway scores show a high-level overview of how these scores change throughout the samples displayed [6]. The Neuropath probe set panel that has 770 genes associated within 24 pathways that can be looked at to embody CNS related paths. The 24 pathways are as followed: Cytokines, Activated Microglia, Angiogenesis, Neurotransmitter Response and Reuptake, Transmitter Release, Growth Factor Signaling, Oxidative Stress, Apoptosis, Chromatin Modification, Unfolded Protein Response, Unfolded Protein Response, Carbohydrate Metabolism, Tissue Integrity, Trophic Factor, Myelination, Lipid Metabolism, Transcription and Splicing, Matrix Remodeling, Neuronal Cytoskeleton, Disease Association, Transmitter Synthesis and

Storage, Autophagy, Vesicle Trafficking, Neuronal Connectivity, Axon and Dendrite Structure

The heatmaps can be read through the probe annotation including a spectrum from blue to orange and gives color to signify whether there is down-regulation, no significance, or up-regulation z-scores within each pathway. By using this technology, it gives the ability to not look at specific genes but rather grouping of genes associated with pathways to understand where we are seeing up or down regulation across our samples and determine if we see significance in the brain due to age, sex, genotype, and/ or treatment.

Results

The results will be discussed in two parts based on how they were analyzed; first looking at the baseline effects between age, sex, and genotype followed by examining the treated effects after IP-LPS allowing us to see immune response in the brain based on age, sex, and genotype from naïve condition. The data will demonstrate that in the basal analysis, sex is the driving factor enveloping the differences we see. Further the data will demonstrate that when comparing inflammatory response instead it is age that is the differentiating factor attributing to the differences we see in the CNS pathway z-scores.

Baseline

At the forefront of this research was the importance of establishing what our genotypes look like at basal level. When approaching our baseline data, we set up one simple question aimed to be answered by our results. At basal level do we see sex differences between WT and Trem2KO genotypes? The data has indeed demonstrated that there are sex differences that can be seen in our naïve samples within the two genotypes. Further simply concluding that yes there are sex differences in the WT genotype, additionally there are sex differences in the Trem2KO genotype. The differences the data will show are in an age- dependent manner and vary within ages. Further it can be seen that sex played a more significant role and attributing to playing an important differentiating factor in comparison to age. To discuss how this conclusion was reached the data will be discussed in terms by the ages and further by the genotype in each of the following sections.

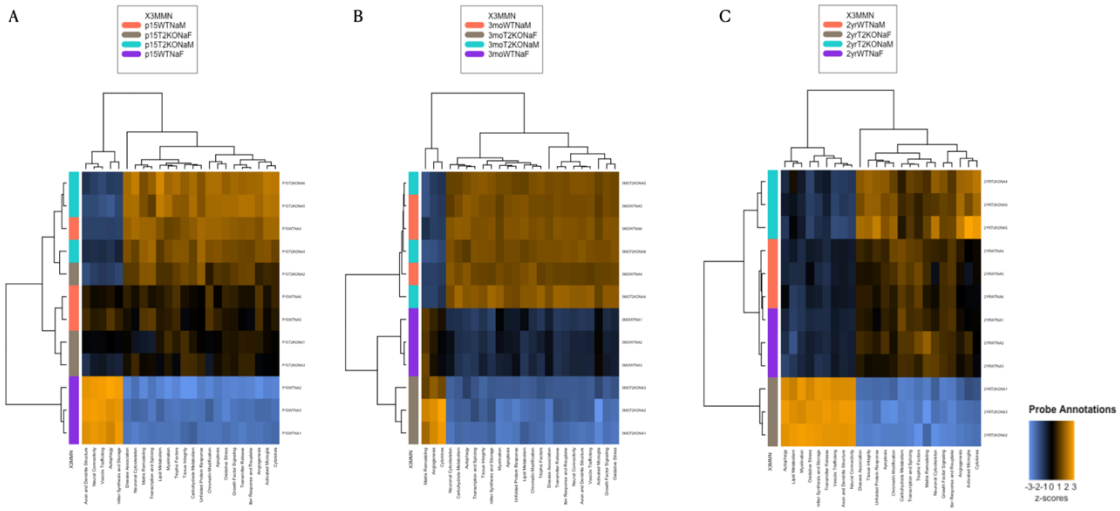

Figure 1 (A, B, C): Heatmaps projected by NanoString Technologies through nSolver Advanced Analysis Software. Analysis n to examine the baseline of samples through direct comparison between sexes within genotypes across three ages. Emulating that at baseline there are sex differences present at all ages, however, vary in genotype and age.
(A): p15 Male and Female Naive WT: p15 Male and Female Naive T2KO; Reference: p15 Male Naive WT. Drawn from Nanostring's Advanced Analysis where we can look at the baseline trend in pathways at p15 age.
(B): 3month Male and Female Naive WT: 3month Male and Female Naive T2KO; Reference: 3month Male Naive WT. Drawn from Nanostring's Advanced Analysis where we can look at the baseline trend in pathways at 3 months of age.
(C): 2year Male and Female Naive WT: 2year Male and Female Naive T2KO; Reference: 2year Male Naive WT. Drawn from Nanostring's Advanced Analysis where we can look at the baseline trend in pathways at 2 years of age.

Baseline at Postnatal 15 Days

In Figure A I ran an Advanced Analysis to look specifically at age p15 samples and what their baseline pathways score signatures show us. In this analysis I look at our two genotypes, WT and Trem2KO, and compare their signatures between males and females. What the data demonstrates is that at baseline there is a profound difference between sexes in the WT genotype. Further this ultimately shows that at the youngest age in our experiment, p15, the WT has clear sex differences that the Trem2KO genotype do not show. Comparing the two sexes the heatmap show exact opposite scoring in terms of up regulated versus down regulated (i.e., where pathways are up-regulated in one sex they are down-regulated in the other sex). Further in the WT, females demonstrate very strong pathway z-scores which can be seen in the bright coloring on the heatmap. The female's pathways signatures were an interesting result that show outlying differences

from the other samples including the Trem2KO samples. The fact that females stand out in a different manner does not end in this data and further will be discussed in the results to follow.

In comparison to the WT, the data suggests little difference in sex within the Trem2KO genotype. While there is slight variability in some of the scores of the pathways there is nothing to suggest significance due to limited z-score differences that can be seen on the heatmap with limited deviation from the black coloration. While it can be somewhat expected that different genotypes will demonstrate different pathway signatures, the main interest in this experiment was the sex difference which we did see in our WT and continued our interest to see if this would be prevalent in the ages to continue in this same genotype. Furthermore, continuing to look to see if the Trem2KO will continue their pattern or will we see sex differences as age increases.

Baseline at 3 Months

In Figure 1B, I continue to run an Advanced Analysis in the same manner as the p15 aiming to look at the baseline in the 3-month-old samples including both WT and Trem2KO. What the heat map data shows are clear sex differences in each our genotypes. Concluding that in the WT genotype there are sex differences between males and females, and within the Trem2KO genotype there are sex differences between males and females. Interestingly, in the heatmap it can also been seen that pathway score signatures are identical within the same sex despite being different genotype (i.e., the female WT and Trem2KO are identical while male WT and Trem2KO are identical). When we look

more closely, examining the WT, the sex differences are visibly clear in all pathways seeing complete opposite up/down z- score signatures throughout. In addition, noting that males are dominantly up-regulated in 21 of the 24 pathways. This signature is the same as the males in the Trem2KO genotype. The 3 pathways that do not follow this same trend and instead are down-regulated include the following: Matrix Remodeling, Angiogenesis, and Cytokines. On the other side the female WT demonstrate signatures that are dominantly down-regulated in the 21 of the 24 pathways. These pathways are the same 21 of 24 that were up-regulated in the males. Furthermore the 3 pathways that were uniquely mentioned to be down-regulated in the males, are in contrast up-regulated in all females both WT and Trem2KO genotypes. This data presents an interesting suggestion that same sex has unifying qualities that brought together similar z-scoring despite their differences in genotype.

Baseline at 2 Years

To conclude the baseline analysis, Figure 1C demonstrates the results of the Advanced Analysis run to examine the two genotypes at the oldest age, 2 years. This heat map showed resemblance to the heat map seen at the p15 age where we see one group demonstrate strong z-scores exemplified in bright coloring. In the p15 that group was the female WT samples, however in the oldest age it is the female Trem2KO that are significantly expressed. Firstly, examining the WT, the data suggests there are no sex differences in the pathways due to the two sexes have unified z-scores in all 24 pathways. In contrast, the Trem2KO samples do show sex differences in complete opposite scoring

from one another. Interesting to note, not only are the Trem2KO females showing strong z-scores, they are demonstrating complete opposite scoring from all other three groups: WT males, WT females, and Trem2KO males.

Baseline Analysis across the three ages

The baseline analysis in Figure 1 through A-C represent what basal levels are in a C57BL/6J WT mouse samples between males and females across p15 (A), 3 months (B), and 2 years (C). Additionally, the data also depicts the basal level pathways signatures in Trem2KO samples between males and females across p15 (A), 3 months (B), and 2 years (C). What can be concluded based on the heatmap data shown is that there are sex differences present at all ages. Those sex differences vary depending on age and depending on the genotype. It was exemplified that WT only demonstrated sex differences in their baseline at p15 and 3-month age. Furthermore, Trem2KO only demonstrated sex differences at 3 months and the 2-year age. Nonetheless, the data evidently showed that sex played a significant impact for the reason that we saw differences within each analysis that were not limited to specific pathways rather in all 24 pathways that showed contrasting signatures.

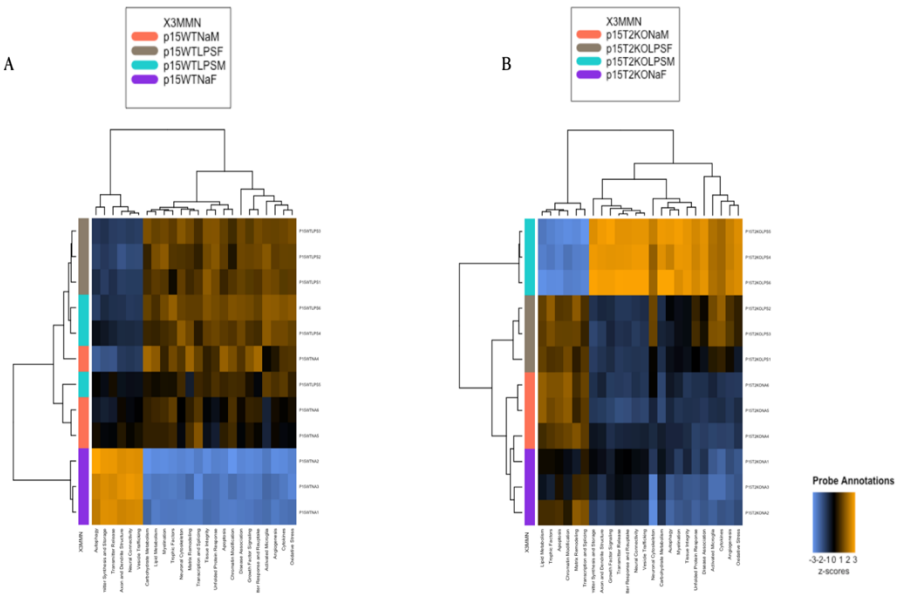

Figure 2 (A and B): Heatmaps demonstrating the effects of LPS between the two genotypes and sexes all at age p15. Analysis demonstrating that depending on genotype, it can be seen that a specific sex will have the most profound impact to the insult.
(A): p15 Male and Female WT Naive: p15 Male and Female WT LPS; Reference: p15 Male WT Naive. Heatmap drawn from the Advanced Analysis represents the impact of LPS from its naive condition in both male and female samples at p15 age.
(B): p15 Male and Female T2KO Naive: p15 Male and Female T2KO LPS; Reference: p15 Male T2KO Naive. Heatmap drawn from the Advanced Analysis represents the impact of LPS from its naive condition in both male and female samples at p15 age.

Analysis of Immune Response in the CNS Across Age and Genotype

Moving from the Baseline Analysis, the next goal was to examine the impacts that an inflammatory response can have on Neuropathological pathways and how those responses differ based on sex between genotypes and age. I approached these comparisons based on age and looking at the two different genotypes between naïve and LPS-stimulated samples. In these analyses I set out to see how sex and age impact the differences in inflammatory immune response we see amongst our different genotypes (Figure 2). The data emulates the following conclusion that age had the greatest impact in the difference we saw based on comparison between naïve and treated. The following sections will discuss the data results by age and genotype

Inflammatory Response in P15 Wild-Type and Trem2KO

In Figure 2A I examined the effects LPS has on the WT genotype between our males and females at our youngest age, p15. Based on the findings from the Advanced Analysis, it can be seen that LPS has a strong impact in the females that affects all pathways in a complete opposite direction from what their naïve state pathways z-scores show. In this same heat map however, males do not show such a profound impact and instead demonstrate little to no variation between naïve and treated pathway scores. Based on these two findings it can be concluded that at p15 age the WT genotype confirms sex differences. Additionally demonstrating that only females were responsive to our LPS stimulant which affected all pathway z-score signatures in the CNS.

In Figure 2B we transition to see the Trem2KO genotype and whether LPS impacts our two sexes at p15. Based on the heat map from this Advanced Analysis remarkably there is also one sex that has a distinct response to LPS demonstrating strong z-scores and coloration. Interestingly the Trem2KO, unlike the WT where female samples showed this significance, instead it is the male sex that were responsive. The females in the Trem2KO genotype show little to no difference in their pathway signatures between naïve and LPS, concluding that LPS does not seem to alter any of the pathway z-scores. Ultimately, it can be said we do see sex differences, notably that the males were affected from LPS based on the contrasting difference from naïve to treated in all pathway signatures.

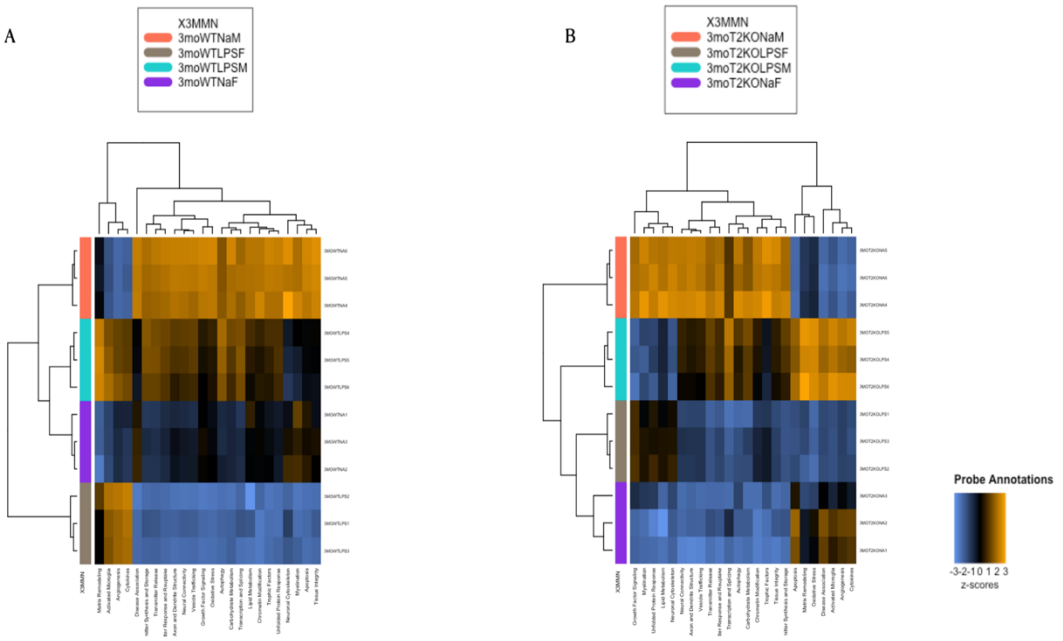

Figure 3: (A and B) Comparison between two heatmaps each showing a specific genotype and its response to LPS from naive condition at 3 months of age. Ultimately showing that depending on genotype, responses are different. In the Wild-Type there are no specific sex differences based on response. In the Trem2KO there are sex differences. LPS stimulant can be seen to impact specific pathways.
(A): 3month Male and Female WT Naive: 3month Male and Female WT LPS; Reference: 3month Male WT Naive. Representation of Wild-Type affects from naive to stimulated condition.
(B): 3month Male and Female T2KO Naive: 3month Male and Female T2KO LPS; Reference: 3month Male T2KO Naive. Representation of Trem2KO affects from naive to stimulated condition.

Inflammatory Response in 3month WT and Trem2KO

In Figure 3 I do a direct comparison looking at both genotypes WT (A) and Trem2KO (B). Interestingly we do not see such a profound impact where a complete opposite direction in all pathways was seen as in the p15 age. What can be seen is that only specific pathways were impacted due to the LPS stimulant. Furthermore, these affected pathways are specific based on genotype. In the WT genotype, there are no sex differences prevalent, rather, the naive of both male and females are unified as are the treated of both male and female. LPS appeared to have upregulated completely the following pathways: Matrix remodeling, activated microglia, angiogenesis, and

cytokines. In contrast LPS played role in down regulated, from baseline, the following: neuronal cytoskeleton, myelination, apoptosis, and tissue integrity.

Next looking at the Trem2KO while we do also see only specific pathways affected, the response is different from the WT counterpart. Another difference is that in Trem2KO at 3 months we do see sex differences. In males, LPS has a profound difference where it down regulates the following pathways: growth factor signaling, myelination, unfolded protein responses, lipid metabolism, and neuronal cytoskeleton. Those mentioned pathways were also significantly impacted by LPS in the females by up regulating them from their naïve states. In addition, females showed down regulated pathway scores to LPS in the following pathways: apoptosis, matrix remodeling, oxidative stress, disease association, activated microglia, angiogenesis, and cytokines. These same pathways are impacted in the opposite manner due to treatment where males are up regulated in those pathways in complete opposite from their naïve states.

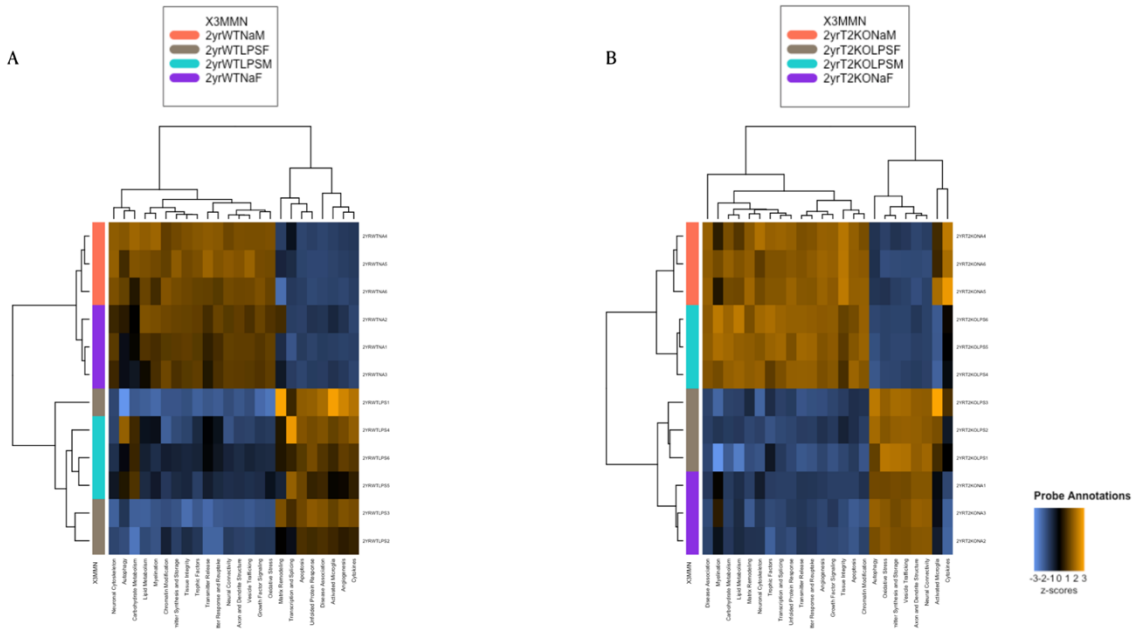

Figure 4 (A and B): Comparison between two heatmaps each showing a specific genotype and its response to LPS from naive condition at 2 years of age. In Wild-Type no sex differences can be seen rather unified responses based on treatment. In the Trem2KO it can be found that there are sex differences yet minimal impact from LPS stimulant.
(A): 2 year Male and Female WT Naive: 2 year Male and Female WT LPS; Reference: 2 year Male WT Naive. Heatmap analysis representation of the Wild-Type genotype between sexes, demonstrate the impact LPS has from naive state in z-scores on pathways.
(B): 2 year Male and Female T2KO Naive: 2 year Male and Female T2KO LPS; Reference: 2 year Male T2KO Naive. Heatmap analysis representation of the Trem2KO genotype between sexes, demonstrate the impact LPS has from naive state in z-scores on pathways.

Inflammatory Response in 2 Year Wild-Type and Trem2KO

The last age in the Data Analysis that we looked at was the 2-year mark. In this analysis we did a direct comparison between the two genotypes (Figure 4). Ultimately finding that within this age we see unified response, however, this unified response is different based on genotype. In the WT we do not see sex differences and instead the pathway scores unify between naive and unify between treated samples. Lastly, looking at the 2-year Trem2KO (4B), the unified response is between the naïve and treated of each sex. The female Trem2KO samples demonstrate exact same pathway signature in 22 out of 24 pathways both at naïve and treated. Similarly in the male Trem2KO samples there too is an exact same pathway signature in 22 out of 24 pathways both in naïve and treated. This result suggests LPS had no impacting effect in the majority of pathways.

Only two pathways did have some responses to LPS, however, whether it was up or down regulated is based on the sex. In females the following were upregulated: cytokines and activated microglia. In the males those mentioned pathways were down regulated due to the LPS stimulant. Ultimately concluding that in the Trem2KO genotype there are sex differences between male and female samples.

Final Conclusions

In the baseline we concluded that age and sex did play factoring roles in the differences that can be seen between genotype. This conclusion is representative based on how all pathway signatures within a genotype demonstrated variability in pathways z-scores scores throughout the three points of ages. This variability can be seen in select pathways based on their age. More notably would be the significant sex differences that persisted significantly within all pathways in Trem2KO genotype at 3months and 2 years, and in WT with near all pathways at age p15 and 3 months. Interestingly, there was no sex difference in WT at 2 years or in Trem2KOs at p15. Sex differences, ultimately, played stronger impact due to the profound differences made among all pathways throughout signature in all 24 paths.

In the second part of the data, we look at the immune response and the impact it may have on the CNS through relevant pathways. In the WT the data showed that in the youngest age females are highly reactive to the insult in all their pathways. Following this genotype through the ages we see no sex differences and similar limited response to LPS at 3 months, lastly at 2 years we also see no sex differences rather a unified response at

naïve (which data showed baselines were exact same) and LPS pathways scoring. We do still see significant change in pathways scoring due to LPS at 2 years, simply that there is no difference in sex at this age. This ultimately shows that inflammatory response reactivity is highest in the Neuro pathways at our youngest and oldest ages and sex differences are only present in the naïve condition, while LPS unifies the responses in our sexes across all three ages. These results emulating that age tends to be the strong driving factor in inflammatory response.

Following the impact of deletion of Trem2 in our mouse model we see that there is a complete sex difference with respect to which sex shows the significant response to LPS, unlike in WT where females showed this, instead males demonstrated high reactivity in all pathways. Females at this age show limited impact to LPS. At 3 months both sexes show impact to LPS which reverts their pathways scores based on their naïve scores. There is sex difference that was prevalent beginning at baseline and continuing even in treated. We do not see unified responses in LPS like the WT displayed. This trend continues at the 2-year age where, again, there is sex difference from naïve condition. The data has demonstrated that LPS only stimulates two pathways from naïve, those are the activated microglia and cytokines pathways. The inflammatory response insinuates a response at p15 and then we see gradual decline in impact to limited impact by 3 years in the genotype. Overall sex impact is greatest at baseline and in p15 age. When it comes to looking at the effects of an inflammatory response it appears age has the greater driving impact in the differences seen.

Discussion

Overall, this complete data set has provided important information about how age and sex give impact and modulate inflammatory responses due to an acute proinflammatory insult. It can be concluded that sex had a great impact in the differences that were seen in our baselines of the two genotypes; whereas age played the greater impact when it came to sensitivity to pathways scoring due to LPS. Furthermore, due to the deletion of Trem2 we saw less reactivity in the CNS as age increased. This could mean that less reactivity to the brain causes a slower response if there is deterioration, or can be implicated to the unsuccessful reduction of neuroinflammation in the local CNS environment. Both implications, nonetheless, are detrimental to a healthy brain and CNS. The deletion of Trem2 can implicate a delayed response and therefore lead to greater worsening and delayed ability to properly repair and return to homeostasis. This can be in conjunction with the literature today that suggests Trem2 has implications in a protective role against neurodegenerative diseases. A study suggested in their data that complere or partial loss of function of Trem2 supports the notion that Trem2 affects the risk for AD by influencing downstream signaling by mediating phagocytosis of cell debris and amyloid plaques, and suppression of inflammatory reactivity [14].

A second important implication this data has provided is the undeniable findings that females are more impacted by Trem2. This was especially seen in the baseline where Trem2KO impacted females at two distinct ages, the youngest p15 and the oldest 2 years. When examining immune response there were suggestive patterns that Trem2 can play

role in suppressing or exciting immune response at both males and females in specific age- dependent manner.

This data provides a very foundational insight to looking at Trem2 and its role in potential immune response and impact to the CNS. A further direction this data can be utilized to look at would begin by looking at specific genes associated with the pathways and see if significance is being caused by a set of genes or potentially just one main that is impacting the entire signature to reflect significance or non-significance.

Limitation

The data and conclusions demonstrated in this paper outline a high overview of how z-scores within pathways are found to change within analysis. Each pathway has an abundance of genes within each that are known to associate within the pathway's profile. With this being said, it is of importance to note that there are limitations in the data. Due to the fact that this is an overview and account for high number of genes as a whole, not specific genes. It can be seen when looking within a specific pathway that may not show significance however specific genes within that pathway do. The same case has been seen vice versa, where pathways that show no significance when looked more closely at the specific genes do demonstrate values of significance. This occurs due to the overview that takes only into account the average of the entirety of the genes as a whole group. This as a result gives opportunity for future direction to begin looking within pathways and look at specific genes and see if certain ones or groups are attributing to the significant or non-significance of a certain pathway.

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