A transient brain endothelial translatome response to endotoxin is associated with mild cognitive changes post-shock in young mice.

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Abstract

Sepsis-associated encephalopathy (SAE) is a common manifestation in septic patients that is associated with increased risk of long-term cognitive impairment. SAE is driven, at least in part, by brain endothelial dysfunction in response to systemic cytokine signaling. However, the mechanisms driving SAE and its consequences remain largely unknown. Here, we performed translating ribosome affinity purification (TRAP) and RNA-sequencing (TRAP-seq) from the brain endothelium to determine the transcriptional changes after an acute endotoxemic (LPS) challenge. We found that LPS induces a strong acute transcriptional response in the brain endothelium that partially correlates with the whole brain transcriptional response and suggested an endothelial-specific hypoxia response. Consistent with a critical role for the IL-6 pathway, loss of the main regulator of this pathway, SOCS3, leads to a broadening of the population of genes responsive to LPS, suggesting that an overactivation of the IL-6/JAK/STAT3 pathway leads to an increased transcriptional response that could explain our prior findings of severe brain injury in these mice. To identify any potential sequelae of this acute response, we performed brain TRAP-seq following a battery of behavioral tests in mice after apparent recovery. We found that the transcriptional response returns to baseline within days post-challenge. Despite the transient nature of the response, we observed that mice that recovered from the endotoxemic shock showed mild, sex-dependent cognitive impairment, suggesting that the acute brain injury led to sustained, non-transcriptional effects. A better understanding of the transcriptional and non-transcriptional changes in response to shock is needed in order to prevent and/or revert the devastating consequences of septic shock.

Introduction

Sepsis-associated encephalopathy (SAE) is a common manifestation in septic patients. It is characterized by diffuse cerebral dysfunction in the absence of a direct brain infection and often clinically manifests as delirium or coma(1-4). Acute encephalopathy occurs approximately in 70% of patients admitted at intensive care units (ICU)(5). Delirium usually resolves with the patient's overall improvement(3). However, occurrence of SAE is associated with increased ICU stay, six-month mortality post-discharge, and higher risk of long-term cognitive impairment and dementia(1, 6). The risks are higher in patients over 65 years old(7) and those with more severe sepsis(2). Patients admitted at the ICU due to shock are at high risk of developing multiorgan failure, in large part due to a dysregulated inflammatory reaction that involves large concentrations of circulating cytokines, a condition usually known as a 'cytokine storm'(8). The mechanisms driving encephalopathy are thought to involve an impaired microvascular blood-brain barrier, coagulopathy, and reduced perfusion, in response to systemic inflammatory cytokines(1, 4, 9-13). Thus, targeting the brain microvasculature response to shock is a promising avenue to prevent or limit SAE. However, we do not fully understand the mechanisms by which the brain endothelium responds to severe systemic inflammatory signals that lead to brain microvascular dysfunction and SAE.

The role of interleukin-6 (IL-6) in systemic inflammatory conditions is well established(14-16). Prior work showed that IL-6 promotes a sustained loss of endothelial barrier function, both directly(17) and as part of an autocrine loop in response to LPS or TNF(18). We previously reported that a single LPS injection led to increased brain vascular permeability 15 hours post-challenge(19). Mice lacking endothelial expression of SOCS3 (SOCS3iEKO), the main negative regulator of the IL-6/JAK/STAT3 pathway, displayed significantly higher brain microvascular leak and organ injury than wild-type (WT) control mice(19), demonstrating a critical role for this pathway in the regulation of the blood-brain barrier in response to systemic inflammation.

The mechanisms driving LPS- and IL-6-induced brain endothelial dysfunction and its consequences, however, remain largely unknown. To begin addressing this mechanism, we sought to perform translating ribosome affinity purification (TRAP)(20) and RNA-sequencing (TRAP-seq) from the brain endothelium to determine the transcriptional changes after an acute challenge. To determine potential sequelae, we performed TRAP-seq following a battery of behavioral tests in mice after apparent recovery. We found that LPS induces a strong acute transcriptional response in the brain endothelium that returns to baseline within days post-challenge. Consistent with a critical role for the IL-6 pathway, SOCS3iEKO mice displayed an exacerbated transcriptional response. Despite the transient transcriptional changes, mice displayed mild, sex-specific cognitive impairment, suggesting lasting effects in brains exposed to a single endotoxemic shock.

Material and Methods

The commercial sources for critical reagents and their catalog numbers are listed in Supplemental Table 1. Supplemental Table 2 lists all primers used. Detailed protocols are provided in the supplemental methods.

Results

Systemic inflammation induces an acute proinflammatory brain microvascular transcriptional response

To study brain endothelial-specific transcriptional response to systemic inflammation, we crossed Rosa26 fsTRAP transgene with mice carrying a cdh5-CreERT2 inducible endothelial Cre driver. After tamoxifen treatment, mice were challenged with a single intraperitoneal bolus of LPS or vehicle

(saline solution). Fifteen hours later, mice were euthanized and whole brains were processed for bulk RNA-seq (whole brain gene expression) and TRAP-seq (endothelial-specific translatome) (Figure 1A). A strong enrichment in endothelial-specific markers and a loss of non-endothelial gene expression demonstrate the efficiency and specificity of the TRAP protocol (Figure 1B). The TRAP-seq response is consistent with endothelial responses measured by single-cell RNA-seq in recent studies of aging and neuroinflammation(21, 22). We then compared our data with published brain TRAP from Tie2-Cre;Rpl22-HA mice(23). Consistent with the increased specificity of the cdh5-CreERT2(24), we found that many genes induced by LPS in the Tie2-Cre-driven TRAP were not altered in the cdh5-CreERT2-driven TRAP data (Supplemental Figure 1). This is likely not due to differences in the LPS challenge, because the total brain RNA-seq demonstrated a strong correlation between the two datasets (Supplemental Figure 1).

As expected, gene set enrichment analysis of the endothelial transcriptional response to LPS demonstrates a strong activation of innate immunity and proinflammatory signaling (Figure 1C). This challenge induced strong neuroinflammation, as determined by increased expression of GFAP, TNF, and many other proinflammatory markers in whole brain (Supplemental Table 3). Although the transcriptional endothelial response to LPS showed a strong correlation with that of the whole brain (Figure 1D), multiple genes altered only in the TRAP-seq dataset suggested a marked endothelial-specific hypoxic response (Supplemental Figure 2).

We then compared the transcriptional response of SOCS3iEKO mice to littermate control mice. SOCS3 deletion on SOCS3iEKO mice was confirmed by the TRAP-seq data (Figure 1E). The highest responsive genes to LPS were only modestly affected by the loss of SOCS3 (Figure 1F), suggesting an already maximal response to this gene subset. However, SOCS3iEKO mice responded with a much wider transcriptional response to LPS (Figure 1G, Supplemental Table 4). Pathway and transcription factor enrichment analysis of the SOCS3iEKO response demonstrated a strong activation of the JAK/STAT pathway and other transcription factors we previously identified as regulated by IL-6 (Supplemental Figure 3). Consistent with a crucial role for IL-6 driving organ damage, the level of expression in the brain endothelium correlated with the disease severity scoring (Figure 1H).

The proinflammatory response quickly returns to baseline levels upon shock resolution

We then sought to determine if this acute LPS challenge led to behavioral deficits. We tracked the disease severity(19, 25) for four days prior to a battery of behavioral tests and brain TRAP-seq (Figure 2A). We performed this assay only in WT mice, since SOCS3iEKO mice do not survive this LPS challenge(19). All mice had completely recovered by day 4 (Figure 2B). Brain TRAP-Seq and whole brain RNA-seq data from mice after recovery from LPS demonstrate the transient nature of the majority of the endothelial transcriptional responses (Figure 2C, Supplemental Table 5). However, a limited inflammatory response to LPS remains, with increased brain endothelial mRNA levels of several proinflammatory genes (Supplemental Table 5). Consistent with the transient nature of the inflammatory response, we detected limited, sex-dependent behavioral changes in recovering mice compared to saline-treated mice. We found that female mice treated with LPS performed poorly in the nest building test (Figure 2D), while LPS-treated males displayed deficits in the Y maze test (Figure 2E). Both males and females showed no changes in the novel object recognition and the open field tests (Figure 2F-G). No sex-dependent baseline differences were observed in saline-treated mice (Figure 2D-G).

Endothelial cells derived from a human brain show a transient transcriptional response to proinflammatory stimuli

We then performed in vitro assays to determine whether the transcriptional response observed in our TRAP data was a direct effect of LPS or IL-6 signaling on endothelial cells. An LPS challenge quickly induced IL6 expression in the immortalized microvascular brain endothelial cell line HCMEC/D3 (Figure 3A) and in cells originally derived from human brain cortex endothelium (HBEC-5i, Supplemental Figure 4). HCMED/D3 cells retain much stronger expression of endothelial markers than HBEC51 cells (Supplemental Figure 5). This response was further increased if cells were challenged with LPS in the presence of the soluble form of the IL-6 receptor subunit gp80 (sIL-6Rα, denoted as LPS+R). Consistent with our previous observations in human primary endothelial cells(18), this co-stimulation allows for an increased transcriptional control, as evidenced by the increased SOCS3 response (Figure 3A, Supplemental Figure 4A). LPS+R also induced an increase in expression of multiple genes identified by our TRAP-seq (Figure 3A). This suggested an IL-6-dependent autocrine signal in response to LPS. To directly assess the effects of IL-6, we challenged these cells with a combination of IL-6 and its soluble receptor (IL-6+R). As expected, this challenge quickly induced an increase in IL6 and SOCS3 (Figure 3B, Supplemental Figure 4B). In support for a direct response to IL-6, the same challenge promoted a significant increase in the expression of CXCL10, NAMPT and CD47 (Figure 3B). Notably, HCMEC/D3 demonstrated a mild but significant response to IL-6 in the absence of recombinant sIL-6Rα (Figure 3B). This is likely due to the increased endogenous sIL-6Rα expression in HCMEC/D3 cells compared to other endothelial cells (Supplemental Figure 5).

Discussion

The mechanisms driving sepsis encephalopathy are thought to involve an impaired microvascular blood brain barrier, hypo- and hyper-coagulation, and reduced perfusion, in response to systemic inflammatory cytokines(1, 4, 9-13). Post-mortem analysis of septic shock decedents shows a pathology consistent with severe brain endotheliopathy, including edema, hemorrhage, microthrombi, and ischemia that is associated with higher IL-6 expression, microglia activation and neuronal apoptosis(26-29). Consistent with this mechanism, multiple experimental studies in small(19, 30) and large(31, 32) animals demonstrate that shock leads to severe brain microvascular dysfunction. Here, we present evidence that an acute inflammatory reaction leads to a strong but transient transcriptional profile in the brain endothelium. While this response is strongly correlated with that of the whole brain, we identified a transcriptional subset that represents an endothelial-specific response. Notably, this subset suggested an acute brain hypoxic event in response to LPS. We had previously shown that this challenge leads to a disruption in the blood-brain barrier that is worsened in mice lacking endothelial expression of the IL-6 signaling pathway inhibitor SOCS3(19). We show here that loss of SOCS3 leads to a broadening of the population of genes responsive to LPS, suggesting that an overactivation of the IL-6/JAK/STAT3 pathway leads to an increased transcriptional response that could explain the severe brain injury in these mice. Notably, in WT mice, this transcriptional response returns essentially to baseline levels only days after the shock. Despite the transient nature of the response, we observed that mice that recovered from the endotoxemic shock showed mild, sex-dependent cognitive impairment, suggesting that the acute brain injury led to sustained, non-transcriptional effects.

There is strong evidence that IL-6 is a causal factor in the development of cognitive impairment (33-38). Much less is known about the role of IL-6 in SAE and its sequelae. A recent systematic review demonstrates increased IL-6 expression in postmortem brains of septic patients when compared to non-septic decedents(27). IL-6 was found to be increased in cerebrospinal fluid (but not in plasma) of patients with all-cause encephalitis(39). In many animal models, systemic IL-6 signaling disrupts blood-brain barrier, promotes coagulopathy, and increases leukocyte infiltration(40). In mice, brain IL-6 is associated with aging(41) and with endotoxin-induced neuroinflammation(42). Consistent with a crucial role, blockade of circulating IL-6 signaling improved survival and cognitive functions in septic(43) and

trauma(44) mouse models. The transcriptional data presented here is consistent with a critical role for IL-6 signaling acting directly on the brain endothelium. LPS induced a strong expression of multiple targets of the IL-6 pathway, including SOCS3 and IL-6 itself, that was observed in both whole brain and endothelial-specific RNA isolates. Mice lacking endothelial expression of SOCS3 displayed a stronger transcriptional response to LPS. Notably, IL-6 expression was undetectable in both WT and SOCS3iEKO saline groups, but SOCS3iEKO mice showed a 4-fold stronger response than WT mice in response to LPS. Bioinformatics analysis of the transcriptional response of SOCS3iEKO mice compared to WT mice confirmed the overactivation of the JAK/STAT3 signaling axis. Moreover, direct IL-6 stimulation of cultured brain endothelial cells induced an increase in expression of multiple proinflammatory genes identified by our TRAP-seq approach. How these changes may affect brain function in shock survivors, however, remains to be determined.

Little is known regarding sex- or gender-specific risks of developing post-intensive care cognitive decline and dementia. Some clinical studies found an association with sex or gender, while others did not(45-49). Despite the endothelial and whole brain transcriptional profile returning to baseline levels after recovery from LPS, we identified moderate, sex-dependent behavioral changes remaining well beyond severity scoring and temperature returned to normal levels. These findings are consistent with sex-specific cognitive deficits in mouse models of brain hypoperfusion(50-52). It is not yet known however if similar processes drive cognitive decline in these two models. Future work is required to dissect the mechanisms driving this sex-specific response.

Legends to figures

Figure 1. Transcriptional response to LPS in the brain endothelium. (A) Schematic of TRAP approach. (B) Ratio of TRAP-seq over whole brain RNA-seq normalized counts obtained from the same brains. (C) Top 20 entries (by normalized enrichment score, NES) of the gene set enrichment analysis of the endothelial transcriptional response to LPS. Shown at the right of each category are the number of enriched genes in the dataset and the total number of genes for each category. (D) Plot showing the correlation between the LPS response (log2 fold change) in whole brain vs brain TRAP. (E) Normalized counts for SOCS3 in brain TRAP and whole brain datasets from saline-treated mice. (F) Heatmap of the 100 most significantly changed genes in the brain endothelium (left) and the corresponding changes in the whole brain (right) of WT and SOCS3iEKO mice. Genes in gray were not detected by whole brain RNA-seq. (G) Volcano plot of the differential expression in response to LPS for either WT or SOCS3iEKO mice (left) and Venn diagram describing the number of differentially expressed genes in response to LPS (right). (H) Correlation of endothelial levels of IL-6 gene expression vs severity score.

Figure 2. Sustained cognitive impairment despite transitory transcriptional changes. (A) Diagram of the experimental timeline. (B) Daily severity score after saline or LPS injection. Two-way ANOVA of repeated measurements. (C) Heatmap of the top 100 most significantly changed genes 15 hours post saline or LPS injection (left) and the corresponding genes 8 days after saline or LPS injection (right). (D-G) Behavioral tests following recovery from LPS. Assays for nest building (D), Y-maze (E), novel object recognition (F) and open field (G) were performed according to the timeline shown in A. Mann Whitney test (n=7—10 per group). Asterisks, $p < 0.05$.

Figure 3. Direct transcriptional regulation by LPS and IL-6 in cultured HCMEC/D3 cells. (A) Confluent HCMEC/D3 cells were treated for two or six hours PBS, PBS+ sIL-6Rα, LPS, or LPS+sIL-6Rα (LPS+R). RT-qPCR was performed to detect gene expression levels. (B) Confluent HCMEC/D3 cells were treated for two or six hours PBS, PBS+ sIL-6Rα, IL-6, or IL-6+sIL-6Rα (IL-6+R). RT-qPCR was performed to detect gene expression levels. Asterisks, $p < 0.05$ (Two-way ANOVA with Sidak post-hoc test against PBS control). Data compiled from three independent experiments performed in duplicate each.

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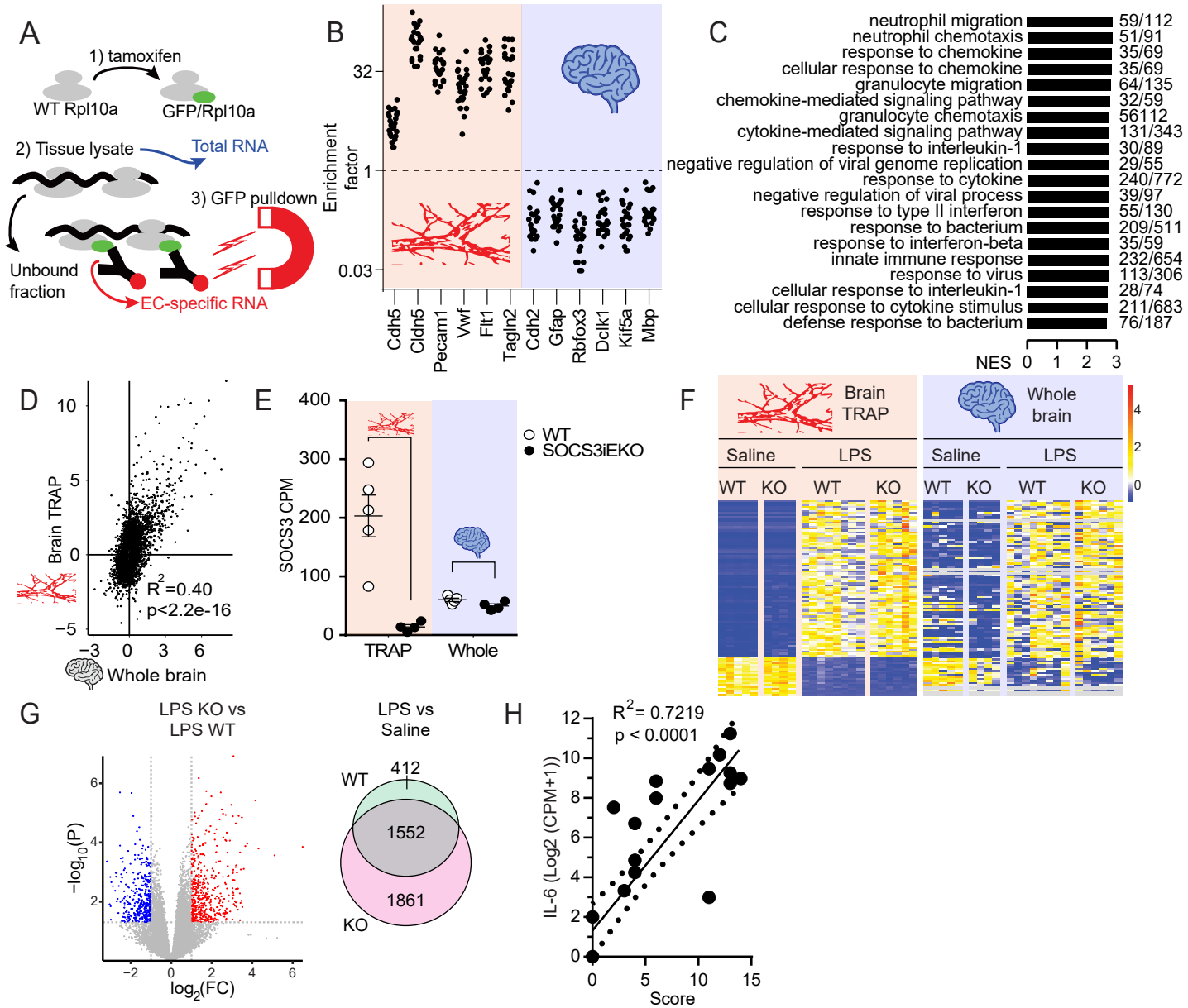
Figure 1


A
1) tamoxifen
WT Rpl10a
GFP/Rpl10a
2) Tissue lysate
Total RNA
3) GFP pulldown
Unbound fraction
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B
Enrichment factor
32
1
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Cdh5
Cldn5
Pecam1
Vwf
Flt1
Tagln2
Cdh2
Gfap
Rbfox3
Dclk1
Kif5a
Mbp
C
neutrophil migration 59/112
neutrophil chemotaxis 51/91
response to chemokine 35/69
cellular response to chemokine 35/69
granulocyte migration 64/135
chemokine-mediated signaling pathway 32/59
granulocyte chemotaxis 56112
cytokine-mediated signaling pathway 131/343
response to interleukin-1 30/89
negative regulation of viral genome replication 29/55
response to cytokine 240/772
negative regulation of viral process 39/97
response to type II interferon 55/130
response to bacterium 209/511
response to interferon-beta 35/59
innate immune response 232/654
response to virus 113/306
cellular response to interleukin-1 28/74
cellular response to cytokine stimulus 211/683
defense response to bacterium 76/187
NES 0 1 2 3
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Brain TRAP
Whole brain
R^2=0.40
p<2.2e-16
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SOCS3 CPM
TRAP
Whole
WT
SOCS3iEKO
F
Brain TRAP
Whole brain
Saline
LPS
WT KO WT KO WT KO WT KO
G
LPS KO vs LPS WT
$-\log_{10}(P)$
$\log_2(FC)$
LPS vs Saline
WT
412
1552
1861
KO
H
R^2= 0.7219
p < 0.0001
IL-6 (Log2 (CPM+1))
Score

Figure 2

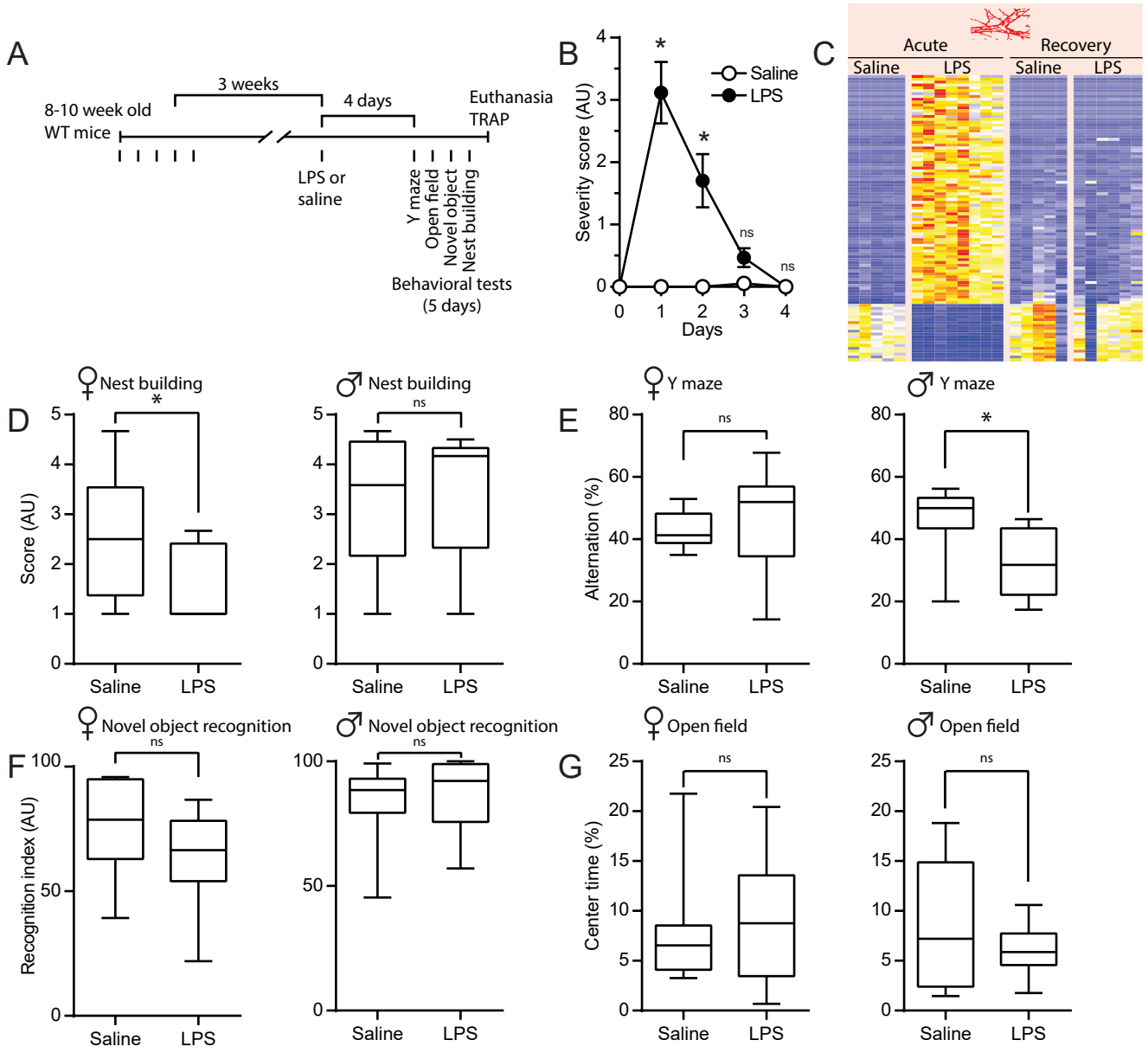
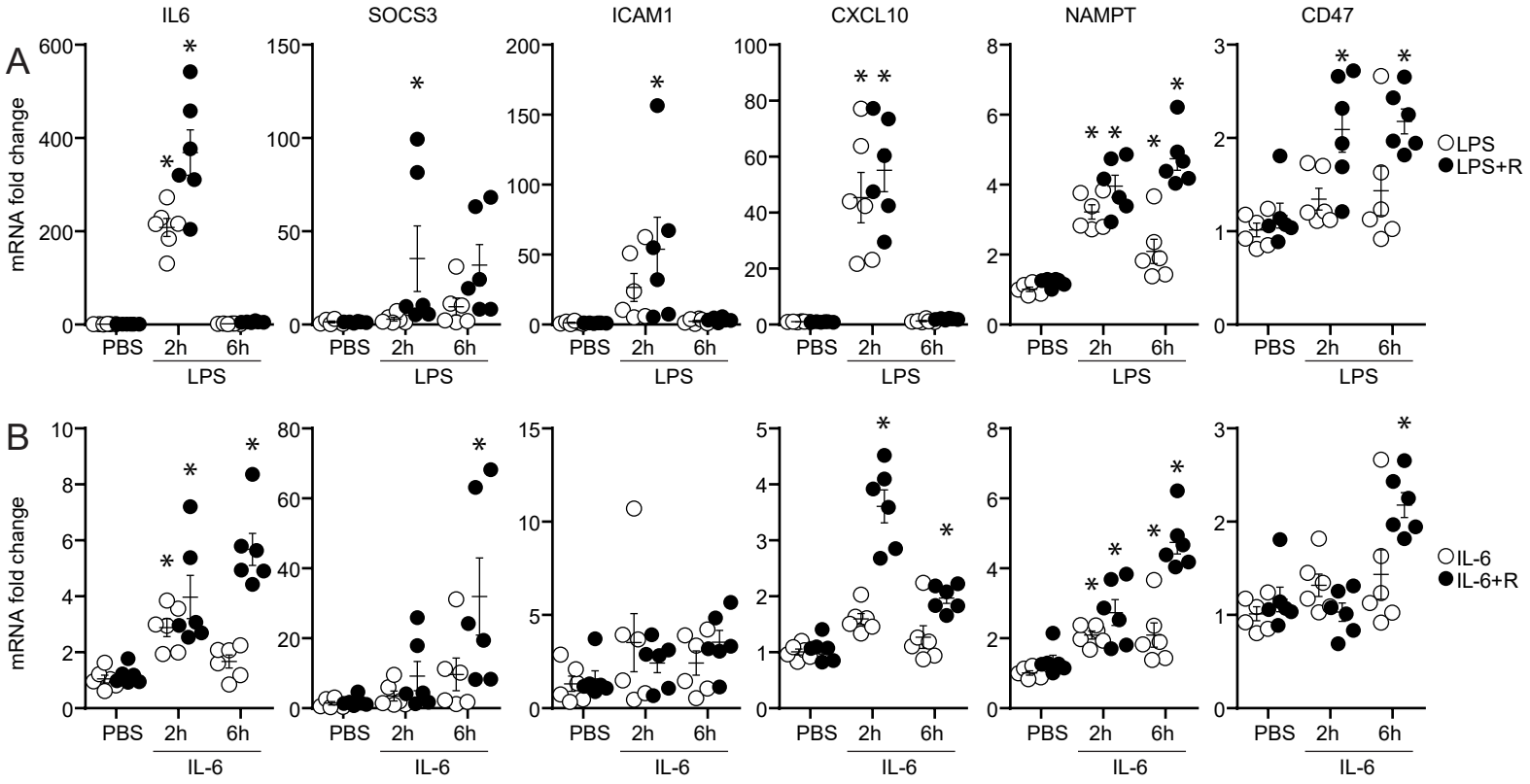

A
3 weeks
4 days
8-10 week old
WT mice
Euthanasia
TRAP
LPS or
saline
Y maze
Open field
Novel object
Nest building
Behavioral tests
(5 days)
B
Saline
LPS
Severity score (AU)
Days
ns
C
Acute
Recovery
Saline
LPS
Saline
LPS
D
♀ Nest building
♂ Nest building
Score (AU)
E
♀ Y maze
♂ Y maze
Alternation (%)
F
♀ Novel object recognition
♂ Novel object recognition
Recognition index (AU)
G
♀ Open field
♂ Open field
Center time (%)

Figure 3


A
B
IL6
SOCS3
ICAM1
CXCL10
NAMPT
CD47
mRNA fold change
PBS
2h
6h
LPS
IL-6
LPS
LPS+R
IL-6
IL-6+R