Sex differences in hippocampal cytokines after systemic immune challenge

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ABSTRACT

Illness or injury causes an inflammatory state consisting of activation of immune cells and increased production of cytokines in the periphery and in the brain, resulting changes in physiological processes, behavior, and cognition. The immune and neuroimmune response consist of a tightly controlled activation and resolution of cytokine networks, the precise patterns of which are determined, in part, by the immune stimulus. Importantly, the pattern of cytokines, rather than the presence of any individual cytokine, determines the functional outcome of immune signaling. In this project, we hypothesized that given sex differences in behavioral responses to immune challenge, the patterns of cytokine activation induced in the hippocampus after a systemic immune challenge differ between males and females. We examined 32 cytokines in the hippocampus and periphery of male and female mice 2, 6, 24, 48, and 168 hours after an acute systemic injection of lipopolysaccharides (LPS; 250μg/kg). All animals showed resolution of the neuroimmune response 168 hours after immune challenge Males and females differed in the specific cytokines activated in the hippocampus, the magnitude of elevation, and the timecourse of activation and resolution of neuroimmune signaling. Briefly, male-specific elevations included IFNγ, CSF1 (M-CSF) and CSF2 (GM-CSF), and the regulatory cytokine IL-10, whereas female-specific activation included the IL-2 family and the regulatory IL-4. Females showed rapid elevation and resolution of the hippocampal immune response, with cytokine levels peaking at 2 and 6 hours after immune challenge. In contrast, males showed slower and more persistent activation, with peaks at 6-24 hours. These findings demonstrate that sex differences in neuroimmune response are not limited to the intensity of the cytokine response, but more importantly differs in the cytokine networks activated. These findings suggest that delineating the broad, sex-specific patterns of cytokine activity in the brain is critical for understanding of the role of neuroimmune signaling in neural function.

INTRODUCTION

In sickness and in health, cytokines in the brain regulate a variety of functions including physiological states, behavior, and synaptic plasticity [1–7]. As such, increased neuroimmune signaling as a consequence of illness or injury causes physiological, behavioral, and cognitive changes including lethargy, changes in affect, memory impairments, and fever [8–10].

There are well-described sex differences in both the peripheral immune response [11–14] and the physiological and behavioral responses to an immune challenge [15,16]. Interestingly, these sex differences are both quantitative, with women and female rodents showing stronger febrile responses, and greater regulation of mood [17–19]; and qualitative, with men and women showing different kinds of responses, including male-specific "sighing" [20] and female-specific changes in pain sensitivity [16]. Such sex differences after peripheral immune challenge suggest that there are sex differences in level or kind of cytokines activated in the brain, and/or that similar cytokines exert different effects on behavior in females and males.

There is strong evidence for both quantitative sex differences in neuroimmune response to a systemic immune challenge, and for qualitatively different roles for neuroimmune cells and cytokines in males and females. Tonelli and colleagues [17] observed that female rats showed greater Interleukin (IL)-6 and Tumor Necrosis Factor (TNF) α gene expression in the hippocampus 24 hours after lipopolysaccharides (LPS) injection. In contrast, males show more IL-1β after LPS [19] and a stronger inflammatory response in the hippocampus after stress [21,22]. Cytokines also play different roles in the brain of males and females. Hippocampal IL-2 impairs neurogenesis only in males [23], whereas IL-13 mediates symptoms in models of multiple sclerosis only in females [24]. Males and females therefore show different functional correlates of neuroimmune signaling, perhaps determined by sex-specific patterns of cytokine activation in the brain.

As in the periphery, IL-1β, IL-6, and TNFα have been particularly well studied for their functional correlates in the brain after many different inflammatory challenges [1,3,25–29]. Despite the consistent activation of these cytokines, different immune challenges lead to unique functional correlates [30,31]. As such, the precise role of individual cytokines likely depends on the exact pattern of immune cells and cytokines activated concurrently [31–33]. Indeed, immune signaling exhibits complex and tightly regulated expansion and resolution of cytokines, a process that is well described in the periphery [33–36]. After the initial challenge, acute phase cytokines TNF and IL-1 rapidly accumulate and stimulate the production of other cytokines such as IL-6 and interferon ɣ (IFNɣ). These, in turn, recruit specific immune cells, and release additional cytokines and chemokines, including the regulatory cytokines IL-10, IL-4, and IL-13 [34]. The precise pattern of cytokine activation and resolution is dependent on several factors: the stimulant, which acute phase cytokines are induced, which immune cells are recruited, what tissue the response is in, and the concurrent endocrine and inflammatory environment [30,33,37]. Such network-dependent patterns of immune signaling means that in the periphery [33,37,38], and in the brain [2,31,39] functional outcomes are determined by both the magnitude of activation and the specific pattern of cytokines induced. Therefore, differences in the cytokine networks activated in the brain in females compared with males are likely critical determinants of sex-specific outcomes after an immune challenge.

In this project, we determined the sex-specific patterns of cytokine activation in the hippocampus after a systemic LPS challenge. We examined 32 cytokines in the hippocampus and serum of males and females from 2 hours to 7 days after an acute, systemic LPS injection. We anticipated that both males and females would show increased cytokine signaling, but that patterns of cytokine activation, kinetics, and magnitude of changes would differ between the sexes. Delineating the patterns of cytokine activation in the brain after a systemic immune

challenge in males and females is essential for understanding how cytokine-dependent signaling modulates behavior and neural functioning in a sex-specific manner.

METHODS

Animals

38 male and 40 female C57Bl6 mice (Harlan Laboratories, Indianapolis, IN), 9-11 weeks old were allowed to habituate to the colony room for at least 5 days prior to injections. Animals were housed individually in standard mouse caging, with *ad lib* access to food and water, a 12:12h light:dark cycle (lights on 7am-7pm) and temperature maintained at 70°F.Individual housing of adult males is required to reduce fighting-induced stress, and is consistent with AAALAC guidelines on management of fighting in mice [40]. To keep social influences constant across animals in both sexes in males and in females, we maintained individual housing in both sexes. Due to independent social structures of both male and female mice [41,42], individual housing is ecologically appropriate for both sexes and does not increase variance in either sex [43]. All procedures were approved by University of Michigan Committee for the Care and Use of Animals.

Lipopolysaccharide Injections

Mice were given single (i.p.) injections of lipopolysaccharides (LPS; E coli, O111:B4; Sigma, St Louis) (250μg/kg in 2mL/kg) or sterile phosphate buffered saline (2mL/kg; PBS). This dose of LPS has previously been demonstrated to result in a robust cytokine response in the hippocampus [44] and memory impairments [45,46] in adult male mice. All animals were monitored after injection for recovery from LPS induced illness.

Tissue Dissections

2 (males n=5; females n=5), 6 (males n=5; females n=5), 24 (males n=5; females n=5), 48 (males n=5; females n=5), or 168 (males n=5; females n=5) hours after LPS or PBS (males n=13;

females n=13) injection, mice were rapidly decapitated, hippocampi immediately dissected and frozen in liquid nitrogen. Trunk blood was collected and allowed to clot at room temperature for approximately 30 minutes, then centrifuged at 1000 rpm for 20 minutes and serum without red blood cells was collected.

Tissue Preparation

Hippocampal tissue was sonicated in low-detergent RIPA buffer (0.01% Triton-X in 1X PBS, with NaVO4, NaF, EDTA, and protease and phosphatase inhibitor (HALT)), centrifuged at 11500rpm, and supernatant collected and stored at -20°C. Bradford assays (Bio-rad Laboratories, Hercules, CA) were used to determine protein concentration of each sample.

Cytokine Assays

32-plex Magplex cytokine assays (EMD Millipore Darmstadt, Germany) [47] were used according to the protocols provided, with minor adaptations for brain tissue. Briefly, 25μL of diluted serum were loaded per well, hippocampal tissue samples were filtered and diluted to concentrations of 1μg/μL prior to loading 25μL of per well. All samples were run in duplicate, and all plates were read on a MagPix (Luminex Corp, Austin TX) machine.

The following cytokines were assayed: CSF1, CSF2, CSF3, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IFNγ, CXCL1, CXCL2, CXCL5, CXCL9, CXCL10, CCL2, CCL3, CCL4, CCL5, CCL11, TNFα, VEGF, LIF, and LIX. Wash was run between each well to prevent build-up of beads and tissue on the plate. Cytokine concentrations were calculated as pg/mg of hippocampal tissue and pg/mL of serum via the native Luminex software.

Statistical Analysis

Statistical analyses were conducted on protein concentration data. PBS treated animals were collapsed into one group for each sex. Two-way multivariate ANOVA was used, with Sex

and Time post injection as factors. Effect size was estimated using partial Eta-squared (η^2p). Post-hoc tests were used to further examine significant effects, with Bonferroni corrections for multiple comparisons. To determine whether males and females differed at baseline, we compared baseline data from both sexes run on the same multiplex plate. As no differences were found, we directly compared changes in individual cytokines between males and females, without the small variations in baseline, we normalized all values to the 0 timepoint (PBS) controls for each sex and compared changes from baseline using these values. Analyses were carried out using SPSS, and all data is expressed as mean ± SEM.

RESULTS

Cytokines in the hippocampus

Of 32 cytokines tested, 27 exhibited a significant elevation in the hippocampus after systemic LPS injection (Main effect of TIME, Table 1), four (LIF, IL-3, IL-5, and IL-15) were not significantly elevated when collapsed across males and females, and CXCL5 (LIX) was excluded as it never reached minimum detectable concentration as defined by the Magplex kit. Post-hoc tests demonstrated rapid activation of IL-1α ($p < 0.01$), IL-2 ($p < 0.001$), IL-4 ($p < 0.01$), IL-6 ($p < 0.001$), IL-9 ($p < 0.001$), IL-12p40 ($p < 0.01$), IL-13 ($p < 0.05$), IL-17 ($p < 0.001$), CSF3 ($p < 0.001$), CXCL1 ($p < 0.001$), CXCL2 ($p < 0.001$), CCL11 ($p < 0.001$) and VEGF ($p < 0.001$) 2 hours after systemic LPS injection. Of these, only IL-4 and CCL11 showed fast resolution with no ongoing increase at the 6 hour timepoint. Activation of CSF1 ($p < 0.001$), CSF2 ($p < 0.001$), TNFα ($p < 0.001$), IFNγ ($p < 0.001$), IL-1β ($p < 0.001$), IL-7 ($p < 0.05$), IL-10 ($p < 0.001$), IL-12p70 ($p < 0.01$), CXCL9 ($p < 0.001$), CXCL10 ($p < 0.001$), CCL2 ($p < 0.001$), and CCL5($p < 0.001$) was first observed 6 hours after LPS. Elevated levels of several cytokines, including CSF2 ($p < 0.01$), TNFα ($p < 0.05$), IFNγ ($p < 0.001$), IL-6 ($p < 0.001$), IL-9 ($p < 0.01$), IL-10 ($p < 0.01$), IL-12p40 ($p < 0.05$), CXCL1 ($p < 0.05$), CXCL9 ($p < 0.001$), CXCL10 ($p < 0.001$), and CCL2 ($p < 0.001$) persisted at least 24 hours

after LPS injection. There were no ongoing elevations in cytokine levels 48 hours after LPS.

Sex differences in activation. Both males and females exhibited a robust neuroimmune response in the hippocampus, and sex differences were observed in specific cytokines activated, the time course of cytokine response, and the magnitude of activation (Fig. 1). In the hippocampus, 20 of 31 cytokines showed differential activation in males and females, via a significant SEX x TIME interaction (see Table 1). IL-7, IL-9, IL-12p70, IL-17, CCL2, CCL4, CCL11, CXCL2 and CXCL5 showed no significant SEX x TIME interaction, but were elevated in the hippocampus in both males and females, whereas IL-5, and LIF were not elevated in either sex. IL-3 and VEGF showed very small effect size for SEX x TIME interaction ($\eta^2_p = 0.18$; $\eta^2_p = 0.15$, respectively). Post-hoc tests were used to determine significant elevations at each timepoint. No significant elevations in males or females were observed at 168 hours. Finally, to determine whether elevations in cytokine level differed between sex at each time point for cytokines that showed significant Sex x Time effects, we used post-hoc tests for data normalized to baseline controls (data shown Fig. 1).

IL-1, TNFα, and IL-6. IL-1α and IL-1β were elevated in the hippocampus after systemic LPS. IL-1β was significantly increased in both males and females, with different time courses [SEX x TIME: $F(5,66) = 8.31$, $p < 0.001$]. Females showed rapid activation 2 hours after LPS injection ($p < 0.05$; m *vs* f: $p < 0.05$), which was resolved by 6 hours ($p = 0.20$). In contrast males showed a later increase, peaking at 6 hours after injection ($p < 0.01$; 24h: $p = 0.39$) (Fig. 2a). IL-1α showed a female-specific increase in the hippocampus 6 and 24 hours after LPS, and resolved by 48 hours [SEX x TIME: $F(5,66) = 9.11$; female 6h: $p < 0.001$; 24h: $p < 0.01$; male 6h: $p = 0.67$ 24h: $p = 0.65$; m *vs* f 6h: $p < 0.001$; 24h: $p < 0.01$].

TNFα showed significant differences in time course of activation in males compared with females [SEX x TIME: $F(5,66) = 6.19$, $p < 0.001$;m *vs* f: $p < 0.01$]. Both sexes showed peak

levels at 6 hours (Female: $p < 0.01$; Male: $p < 0.001$; m *vs* f: $p = 0.18$), however this response persisted at least 24 hours only in male mice (Male: $p < 0.01$; Female: $p = 1.00$; m *vs* f: $p < 0.05$) (Fig. 2b).

IL-6, like IL-1β, exhibited significant increases in the hippocampi of both male and female mice, with sex differences in time course [SEX x TIME: $F(5,66) = 3.62, p < 0.01$]. Again, females mounted earlier responses, with increased IL-6 observed at 2-6 hours post injection, that had returned to baseline by 24 hours (2h: $p < 0.001$; 6h: $p < 0.01$; 24h: $p = 0.853$; 2h m *vs* f: $p < 0.001$). Males showed elevations at 6 hours that persisted for at least 24 hours (2h: $p = 0.100$; 6h $p < 0.001$; 24h: $p < 0.01$) (Fig. 2c).

IL-10, IL-4, IL-13. We also observed striking sex differences in IL-10, IL-4, and IL-13 (Fig. 3). Males showed significant activation of IL-10 6 and 24 hours after LPS injection [$F(5,66) = 5.48$, $p < 0.001$; Males 6h: $p < 0.001$, 24h: $p < 0.01$], whereas females showed only a smaller elevation at 6h (Females 6h: $p < 0.01$; 24h $p = 0.36$; m *vs* f 6h: $p < 0.01$; 24h: $p < 0.01$) (Fig. 3a). In contrast, IL-4 was increased only in females [SEX x TIME: $F(5,66) = 5.33$, $p < 0.001$; Females 2h: $p < 0.001$; Males: $p = 0.21$; m *vs* f: $p < 0.001$] (Fig. 3b). Finally IL-13, an "IL-4-like cytokine" was differentially activated in females and males over time [TIME: $F(5,66) = 16.52$, $p < 0.001$; SEX x Time $F(5,66) = 2.12$, $p < 0.001$]. IL-13, like IL-6 and IL-1β, was induced earlier in females, peaked at 2 hours and returned to baseline by 6 hours post LPS (2h: $p < 0.01$; 6h: $p = 0.88$; m *vs* f 2h: $p < 0.01$), whereas in males, IL-13 levels increased later, and stayed elevated for longer (6h: $p < 0.00$; 24h $p = 0.018$; m *vs* f 6h: $p < 0.01$; 24h $p < 0.001$) (Fig. 3c).

IL-2 family cytokines (IL-2, IL-15, IL-9). The IL-2 family of cytokines was also activated in both sexes, with a bias towards activation of IL-2 in male animals and IL-15 in females (Fig. 4). In males and females, IL-2 was elevated 6 hours after LPS injection, and but this persisted only in males [SEX x TIME: $F(5,66) = 4.15$, $p <0.01$; Females 6h: $p < 0.05$; 24h: $p < 0.383$; Males 6h:

p < 0.01; 24h p < 0.05; 48h $p < 0.01$; m *vs* f 6h: $p = 0.14$; 48h: $p < 0.05$] (Fig. 4a). In contrast, IL-15 was elevated at 2 hours [SEX x TIME: $F(5,66) = 6.23$, $p < 0.001$; Females 2h: $p < 0.01$; Males 2h: $p = 0.24$; m *vs* f 2h: $p < 0.001$] (Fig. 4b). IL-9 was significantly increased in both males and females at 6, 24, and 48 hours after LPS [TIME: $F(5,66) = 16.52$, $p < 0.001$; SEX x Time $F(5,66) = 2.12$, $p > 0.05$].

IFNɣ, CXCL9, and CXCL10. IFNɣ showed male-specific activation in the hippocampus [SEX x TIME $F(5,66) = 27.37$, $p < 0.001$]. Males, but not females, showed elevations at 6 hours and 24 hours (Males 6h: $p < 0.001$; 24h $p < 0.001$) after LPS injection (Fig. 5a).

The CXCR3 ligands CXCL9 (MIG) and CXCL10 (IFN gamma induced protein 10; IP-10) were increased in both males and females [CXCL9 TIME: $F(5,66) = 40.47$, $p < 0.001$;CXCL10 TIME: $F(5,66) = 115.95$, $p < 0.001$]. Both CXCL9 and 10 showed sex differences in activation with higher elevations in males compared to females [SEX x TIME: $F(5,66) = 7.50$, $p < 0.001$; CXCL10 SEX x TIME: $F(5,66) = 5.62$, $p < 0.001$]. CXCL9 exhibited pronounced differences in time course of activation between males and females, with females showing activation of CXCL9 only at 6 hours (6h: $p < 0.001$; 24h: $p = 0.33$), whereas CXCL9 persisted for at least 24 hours in males (6h: $p < 0.001$; 24h: $p < 0.01$; 48h: $p = 0.50$) (Fig. 5b). CXCL10 was elevated in both sexes at 6h and 24h (all $p < 0.001$) and persisted in males at 48h (Males: $p < 0.05$; Females $p = 0.91$), nevertheless, CXCL10 showed greater elevations than females at all time points (m *vs* f 6h: $p < 0.001$; 24h: $p < 0.001$; 48h: $p < 0.05$) (Fig. 5c).

Colony Stimulation Factor (CSF1, CSF2, CSF3). All three CSF family members show significant elevation after LPS and differential activation in males and females [CSF1 TIME: $F(5,66) = 17.59$, $p < 0.001$; SEX x TIME: $F(5,66) = 9.40$, $p < 0.001$; CSF2 TIME: $F(5,66) = 16.41$, $p < 0.001$; SEX x TIME: $F(5,66) = 4.78$, $p < 0.001$; CSF3 TIME: $F(5,66) = 81.25$; $p < 0.001$] (Fig. 6).

CSF1 and CSF2 showed male-specific activation, and were significantly elevated 6 and 24 hours after LPS injection [CSF1 2h: $p = 0.972$, 6h: $p < 0.001$, 24h: $p < 0.001$; CSF2 2h: $p = 0.78$, 6h:

$p < 0.001$; 24h: $p < 0.005$], whereas females showed no change in either CSF1 or CSF2 at any time point (all $p > 0.1$) (Fig. 6a,b). In contrast, females showed elevations in CSF3 at both 2 and 6 hours after LPS injection [2h: $p = 0.004$; 6h; $p < 0.001$; 24h: $p = 0.994$], and males show an increase in CSF3 only at 6h post LPS [2h: $p = 0.127$; 6h; $p < 0.001$; 24h: $p = 0.223$], although to a lesser degree than in females (m vs f: $p < 0.001$) (Fig. 6c).

CXCR2 Ligands (CXCL1 and CXCL2, Gro family). CXCL1 and CXCL2 were strongly activated in the hippocampus in both males and females after LPS injection (Fig. 7). CXCL1 showed significant sex differences in activation [SEX x TIME $F(5,66) = 25.50$, $p < 0.001$]. Both males and females showed significant elevations at 2 hours [all $p < 0.001$] and 6 hours (all $p < 0.001$) but only males showed persisted elevations 24 hours after LPS [Males: $p < 0.01$; Females: $p = 0.11$]; nevertheless females showed significantly greater activation compared with males at 2 and 6 hours after LPS (m *vs* f 2h: $p < 0.001$; 6h: $p < 0.001$) (Fig. 7a). CXCL2 was significantly elevated 2 and 6 hours after LPS with no difference between sexes [TIME: $F(5,66) = 17.93$, $p < 0.001$; SEX x TIME: $F(5,66) = 1.489$, $p = 0.205$; Males 2h: $p < 0.01$; 6h: $p < 0.01$; Females 2h: $p < 0.001$; 6h: $p < 0.001$] (Fig. 7b).

CCR5 Ligands (CCL3, CCL4, CCL5). The C-C motif chemokines CCL3 and CCL5 also showed sex differences in activity [SEX x TIME: $F(5,66) = 19.01$, $p < 0.001$]. CCL3 and CCL5 were only increased in females with a small but significant increase in CCL3 2 hours after LPS (means ± SEM: PBS: 17.07 ± 1.73 vs 2h: 24.2 ± 1.48; $p < 0.05$), and a 2-fold increase in CCL5 6 hours after LPS (means ± SEM: PBS: 2.35 ± 0.13 vs 6h: 4.38 ± 0.49; $p < 0.001$). CCL4 was not differentially elevated in either sex (Table 1).

Peripheral cytokines

Of the 32 cytokines tested, 26 exhibited a significant elevation in the periphery after systemic LPS injection (Main effect of TIME, Table 1), six (IL-2, IL-9, CSF1, CSF3, CXCL5, and

VEGF) were not significantly elevated when collapsed across males and females (Table 2). Compared with hippocampal cytokines, activation of peripheral cytokines was more robust, with stronger effect sizes and more rapid induction and resolution after LPS. Post-hoc tests showed that independent of sex, all but one activated cytokine was upregulated 2 hours after LPS (all $p < 0.001$), with CXCL9 upregulated only after 6 hours (2h: $p = 0.696$; 6h: $p < 0.001$). At 6 hours post LPS, CSF2 ($p < 0.001$), TNFα ($p < 0.05$), IL-1β ($p < 0.001$), IL-10 ($p < 0.001$), IL-12p40 ($p < 0.001$), CXCL10 ($p < 0.001$), CCL2 ($p < 0.001$), CCL3 ($p < 0.001$), and CCL5 ($p < 0.001$) remained elevated. With the exception of CXCL9 (24h: $p < 0.001$) and CSF2 (24h: $p < 0.05$) all cytokines had returned to baseline by 24 hours post LPS injection.

Sex differences in activation of serum cytokines. In the serum, 7 of 32 cytokines, IL-1β, CCL3, CCL4, CCL5, CCL11, CXCL2, and CXCL10, showed differential activation in males and females, with a significant SEX x TIME interaction (see Table 2). CCL11 showed very small effect size for SEX x TIME interaction ($\eta^2_p = 0.18$) and was not further examined. Unlike hippocampal cytokines that showed sex differences in magnitude, time course, and pattern of cytokines, serum cytokines only showed sex differences in magnitude of activation. Furthermore, relatively few cytokines in serum compared with hippocampus showed sex differences in activation after LPS injection.

IL-1β was significantly elevated at 2 and 6 hours after LPS injection in both sexes [TIME: $F(5,66) = 88.05$, $p < 0.001$; SEX x TIME: $F(5,66) = 4.25$, $p < 0.001$; 2h: all $p < 0.001$; 6h: all $p < 0.001$), with significantly greater levels in females at 6 hours compared with males ($p < 0.05$) (Fig. 8a). In contrast, TNFα was elevated 2 and 6 hours after LPS [TIME: $F(5,66) = 230.87$, $p < 0.001$; 2h: $p < 0.001$; 6h: $p = 0.018$] (Fig. 8b) and IL-6 at 2 hours after LPS injection [TIME: $F(5,66) = 230.87$, $p < 0.001$; 2h: $p < 0.001$] with no differences between males and females [SEX x TIME: TNFα: $F(5,66) < 1$; IL-6:$F(5,66) = 1.33$; $p = 0.09$] (Fig. 8c).

CXCL2 was elevated in both sexes at 2 hours [TIME: $F(5,66) = 213.58$; $p < 0.001$; SEX x TIME: $F(5,66) = 3.70$, $p = 0.005$; 2h: all $p < 0.001$), with stronger activation in females compared with males ($p < 0.001$) (Fig. 8d). In contrast, CXCL10 was elevated in both sexes at 2 and 6 hours [TIME: $F(5,66) = 64.82$; $p < 0.001$; SEX x TIME: $F(5,66) = 4.27$, $p = 0.002$; 2h: all $p < 0.001$; 6h: all $p < 0.001$], with stronger activation in males than females 6 hours after LPS injection ($p < 0.001$) (Fig. 8e).

The most striking sex differences were observed in the CCR5 ligands, CCL3, CCL4 and CCL5. Both males and females showed activation of CCL3 in serum 2 hours after LPS [TIME: $F(5,66) = 124.39$; $p < 0.001$; SEX x TIME: $F(5,66) = 3.39$, $p = 0.009$; 2h: male: $p < 0.001$; female $p < 0.001$] with stronger levels of CCL3 in females ($p < 0.01$). In contrast, only males showed activation 6 hours after LPS (6h: male $p < 0.01$; female $p = 0.21$), and this activation remained higher than that of females 24 hours after LPS ($p < 0.05$) (Fig. 8f). In addition, CCL4 [TIME: $F(5,66) = 25.10$; $p < 0.001$; SEX x TIME: $F(5,66) = 5.67$, $p = 0.009$; 2h: male: $p < 0.01$; female $p < 0.001$] and CCL5 [TIME: $F(5,66) = 32.56$; $p < 0.001$; SEX x TIME: $F(5,66) = 10.08$, $p < 0.001$; 6h: male: $p < 0.001$; female $p = 0.007$] showed opposing patterns, with higher CCL4 levels in females compared with males 2 hours after LPS ($p < 0.001$) (Fig. 8g); and lower levels of CCL5 6 hours after injection ($p < 0.001$) (Fig. 8h).

DISCUSSION

Here we demonstrated activation and regulation of a broad network of cytokines in the hippocampus after a systemic immune challenge in females and males. These cytokines included CSF3, the IFNɣ activated proteins CXCL9 and 10, CXCL1 and 2, and C-C motif chemokines including CCL2-5 and CCL11, as well as interleukins IL-1, IL-9, IL-12, and IL-13 in both males and females. Consistent with previous studies [23,48,49], the more commonly examined inflammatory targets IL-1β, IL-6, and TNFα were elevated to a lesser degree in the

hippocampus than in the periphery after LPS. In contrast, cytokines in other pathways, notably CXCL9, CXCL10, and CSF3 showed robust activation in the hippocampus. These findings demonstrate that a broad network of cytokines and chemokines is activated in the brain after systemic LPS injection.

This study also demonstrated important sex differences in activation of hippocampal cytokine networks. Acute systemic LPS injection resulted in different cytokines produced, different magnitudes of activation, and different rates of production and resolution of neuroimmune signaling in the hippocampus of males compared with females. Sex-specific activation of cytokines was evident across several families of cytokines including the CSF and IL-2 families of cytokines, together with IL-10, IL-4, and IL-13. Male-specific activation was observed for CSF1 and CSF2, IFNγ, and IL-10, with stronger activation in CXCL9 and 10. In contrast, female-specific activation was observed for IL-2 and IL-15, CCL3 and 5, IL-1α and IL-4, with stronger activation of CSF3 and CXCL1. These findings expand on prior work demonstrating sex differences in hippocampal IL-1β [19] after systemic LPS. Our results also suggest that the recent demonstrations of sex differences in neural activity [50] and serotonergic signaling [51] after LPS, are potentially driven by sex-specific patterns of cytokine signaling. How these differential patterns of cytokine activation correlate with sex differences in the cognitive and behavioral effects of immune challenge remains unknown.

Males and females also exhibited distinct kinetics of hippocampal cytokine regulation after systemic LPS (Fig. 1). Females showed a rapid elevation of cytokines, peaking at 2-6 hours, with resolution of cytokine signaling within 24 hours. In contrast, males showed slower activation, with all cytokines elevated at 6 hours and most persisting for at least 24 hours. This finding is consistent with a recent report demonstrating that 24 hours after systemic LPS, females but not males show elevated IL-1β in hippocampus [19]. Faster neuroimmune

activation and resolution is consistent with more efficient immune response by females compared with males in response to toll like receptor (TLR) activation in the periphery [52].

Cytokine activation was slower and more persistent in the hippocampus compared with the periphery. Unlike the differences in time course, pattern and magnitude in hippocampal cytokines, serum cytokines of males and females differed primarily in magnitude. In the periphery, both sexes showed elevated levels of the same cytokines, but females had stronger activation of IL-1β, CXCL2, CCL3 and CCL4, whereas males showed stronger activation of CCL5 and CXCL10. That cytokine activation, and sex-specific patterns of cytokines differ between trunk blood and hippocampus strongly suggests that the cytokine activation in the brain is not simply a recapitulation (or artifact) of circulating cytokine levels, but rather represents central processes of immune activation and regulation.

Sex differences in patterns of hippocampal cytokine production is consistent with previous findings demonstrating sex and sex-hormone influences on neuroimmune cells both *in vitro* and *in vivo* [53–57]. In addition, microglia from female mice show increased IL-4 but not IL-10 levels, and those from male mice show the opposite pattern –increased IL-10 but not IL-4— after ischemic stroke [58]. Our findings fit the previously observed patterns, notably that males have a greater IL-10 response compared with females [59] and that IL-2 [23] and IL-13 [24] play different roles in the hippocampus in males compared with females. Our data extend these findings to show differential regulation of CSF-family cytokines, IFNɣ and CXCL9 and 10, IL-4 and IL-13, CXCR2 ligands, and CCR5 ligands in the hippocampus.

Sex differences in patterns of cytokines have implications for what neuroimmune cells are recruited in males compared with females. For example, sex differences in IL-4, IL-13, and IFNɣ are of particular interest for their differential roles in microglia activation. IFNɣ has been proposed to cause microglia polarization to a M1-like ("inflammatory") pattern of cytokine

response, and IL-4/IL-13 towards M2-like ("alternative") microglial response [60], similar to macrophage M1/M2 states [61]. The male-specific increase in CSF2 may contribute to the more persistent inflammatory response in males [62], and in females, the short-lasting IL-1β, IL-6 and TNFα profile suggests rapid activation of regulatory processes. Although M1/M2 polarization is likely an oversimplication of microglial behavior [63], this framework maybe useful for conceptualizing how differential patterns of cytokine responses in males and females may be induced and maintained.

Astrocytes, mast cells, eosinophils, leukocytes and macrophages are actively involved in cytokine production during a neuroimmune response and likely contribute to sex differences in cytokine network dynamics. For example, astrocytes show sex-specificity of cytokine release in response to LPS or injury [54,56]. Furthermore, CXCL1 and CXCL2 play key roles in recruitment of immune cells including leukocytes [64] and neutrophils [65] during neuroinflammation. The greater activation of CXCL1 in females compared to males suggests that differential immune cell recruitment and activation may be both cause and consequence of sex differences in cytokine network activation. The precise, sex-specific patterns of recruitment and cytokine release in the brain from microglia, astrocytes and other infiltrating immune cells are yet to be determined.

Several other mechanisms likely contribute to differential cytokine network dynamics in male and females. Coupling of immune-related receptors and intracellular signaling is critical to subsequent responses, and many studies show sex-specificity of these pathways. For example, males show higher numbers of Toll-like-receptor (TLR) 4, and differential regulation of downstream signaling pathways [66–68]. Given that LPS activates immune responses *via* interactions with TLR4, this is a central candidate for sex differences in rapid effects of LPS.

Cytokine receptor function also depends on the patterns of cytokines active at any point

in time, in part due to binding sites for multiple cytokines. For example, both IL-1α and IL-1β (together with antagonist IL-1ra) bind to IL-1RI [69]; IL-4 and IL-13 interact with IL-4Rα [70]; IL-2 and IL-15 are ligands for IL-2Rβ complex [71]; and CXCL9 and CXCL10 are both CXCR3 ligands [72]. This functional redundancy suggests that some sex differences in cytokine levels may be compensated for via activation of cytokines with similar downstream effectors. For example, IL-13 activation in males may partially compensate for the lack of IL-4 upregulation observed in this study. We also observed differences between families of cytokines. IL-2 and IL-15, for example, were only activated in females, suggesting that different subsets of downstream signal transduction pathways are engaged in males and females. Differential activation of downstream effectors may thus mediate sex differences in neural effects and behavioral outcomes of inflammation.

In this project, we demonstrated that a systemic immune challenge activates broad sex-specific cytokines profiles from multiple families in C57Bl6 mice. This hippocampal inflammatory response shows sex differences in the families of cytokines activated, the magnitude of activation, and the time course of activation and regulation of cytokine signaling (Fig. 1). Different patterns of cytokines mediate differential cell recruitment, microglia polarization, and intracellular signaling, thereby leading to very different outcomes of signaling in males and females. The function of classic inflammatory cytokines, therefore, will be better understood within the context of their downstream immune effectors in each sex. Delineating network dynamics of cytokine signaling, and understanding the interactions and relationships between individual cytokines in males and in females will thereby provide a new framework for understanding sex-biased susceptibility to psychiatric and neurological disorders.

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CYTOKINE	MAIN EFFECT: TIME			INTERACTION: SEX x TIME		
Hippocampal cytokines differentially activated in males and females						
IL-1α	***	$F_{(5,66)} = 13.09$	$\eta^2_p = 0.50$	***	$F_{(5,66)} = 9.11$	$\eta^2_p = 0.41$
IL-1β	***	$F_{(5,66)} = 6.93$	$\eta^2_p = 0.34$	***	$F_{(5,66)} = 8.31$	$\eta^2_p = 0.39$
TNFα	***	$F_{(5,66)} = 12.46$	$\eta^2_p = 0.486$	***	$F_{(5,66)} = 6.19$	$\eta^2_p = 0.32$
IL-6	***	$F_{(5,66)} = 30.19$	$\eta^2_p = 0.70$	**	$F_{(5,66)} = 3.62$	$\eta^2_p = 0.22$
IL-10	***	$F_{(5,66)} = 20.21$	$\eta^2_p = 0.60$	***	$F_{(5,66)} = 5.48$	$\eta^2_p = 0.29$
IL-4	***	$F_{(5,66)} = 13.83$	$\eta^2_p = 0.51$	***	$F_{(5,66)} = 5.33$	$\eta^2_p = 0.29$
IL-13	***	$F_{(5,66)} = 23.28$	$\eta^2_p = 0.64$	***	$F_{(5,66)} = 2.12$	$\eta^2_p = 0.42$
IL-2	***	$F_{(5,66)} = 13.88$	$\eta^2_p = 0.51$	**	$F_{(5,66)} = 4.15$	$\eta^2_p = 0.24$
IL-15	n.s.			***	$F_{(5,66)} = 6.23$	$\eta^2_p = 0.32$
IFNγ	***	$F_{(5,66)} = 33.36$	$\eta^2_p = 0.72$	***	$F_{(5,66)} = 27.37$	$\eta^2_p = 0.68$
CXCL9 (MIG)	***	$F_{(5,66)} = 40.47$	$\eta^2_p = 0.75$	***	$F_{(5,66)} = 7.50$	$\eta^2_p = 0.36$
CXCL10 (IP-10)	***	$F_{(5,66)} = 115.95$	$\eta^2_p = 0.90$	***	$F_{(5,66)} = 5.62$	$\eta^2_p = 0.30$
CSF1 (M-CSF)	***	$F_{(5,66)} = 17.49$	$\eta^2_p = 0.57$	***	$F_{(5,66)} = 9.40$	$\eta^2_p = 0.42$
CSF2 (GM-CSF)	***	$F_{(5,66)} = 16.41$	$\eta^2_p = 0.55$	**	$F_{(5,66)} = 4.78$	$\eta^2_p = 0.27$
CSF3 (G-CSF)	***	$F_{(5,66)} = 81.25$	$\eta^2_p = 0.86$	***	$F_{(5,66)} = 27.57$	$\eta^2_p = 0.68$
CXCL1 (KC)	***	$F_{(5,66)} = 103.11$	$\eta^2_p = .89$	***	$F_{(5,66)} = 25.50$	$\eta^2_p = 0.66$
CCL3 (MIP1α)	*	$F_{(5,66)} = 2.62$	$\eta^2_p = 0.17$	***	$F_{(5,66)} = 19.01$	$\eta^2_p = 0.59$
CCL5 (RANTES)	***	$F_{(5,66)} = 8.97$	$\eta^2_p = 0.41$	***	$F_{(5,66)} = 13.09$	$\eta^2_p = 0.50$
IL-12p40	***	$F_{(5,66)} = 16.12$	$\eta^2_p = 0.55$	**	$F_{(5,66)} = 3.94$	$\eta^2_p = 0.23$
VEGF	***	$F_{(5,66)} = 15.22$	$\eta^2_p = 0.54$	*	$F_{(5,66)} = 2.34$	$\eta^2_p = 0.15$
IL-3	n.s.			*	$F_{(5,66)} = 2.81$	$\eta^2_p = 0.18$
Hippocampal cytokines activated in both sexes						
IL-9	***	$F_{(5,66)} = 16.52$	$\eta^2_p = 0.56$	n.s.		
CCL2 (MCP1)	***	$F_{(5,66)} = 31.92$	$\eta^2_p = 0.71$	n.s.		
CCL11 (eotaxin)	***	$F_{(5,66)} = 17.06$	$\eta^2_p = 0.56$	n.s.		
CCL4 (MIP1β)	***	$F_{(5,66)} = 6.12$	$\eta^2_p = 0.32$	n.s.		
CXCL2 (MIP2α)	***	$F_{(5,66)} = 17.93$	$\eta^2_p = 0.58$	n.s.		
IL-12p70	***	$F_{(5,66)} = 8.24$	$\eta^2_p = 0.38$	n.s.		
IL-17	***	$F_{(5,66)} = 17.12$	$\eta^2_p = 0.57$	n.s.		
IL-7	**	$F_{(5,66)} = 4.15$	$\eta^2_p = 0.24$	n.s.		

Table 1. Hippocampal cytokines: Statistical analysis. Overall multivariate ANOVA results for the Main effect of TIME and the interaction effect (SEX x TIME). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

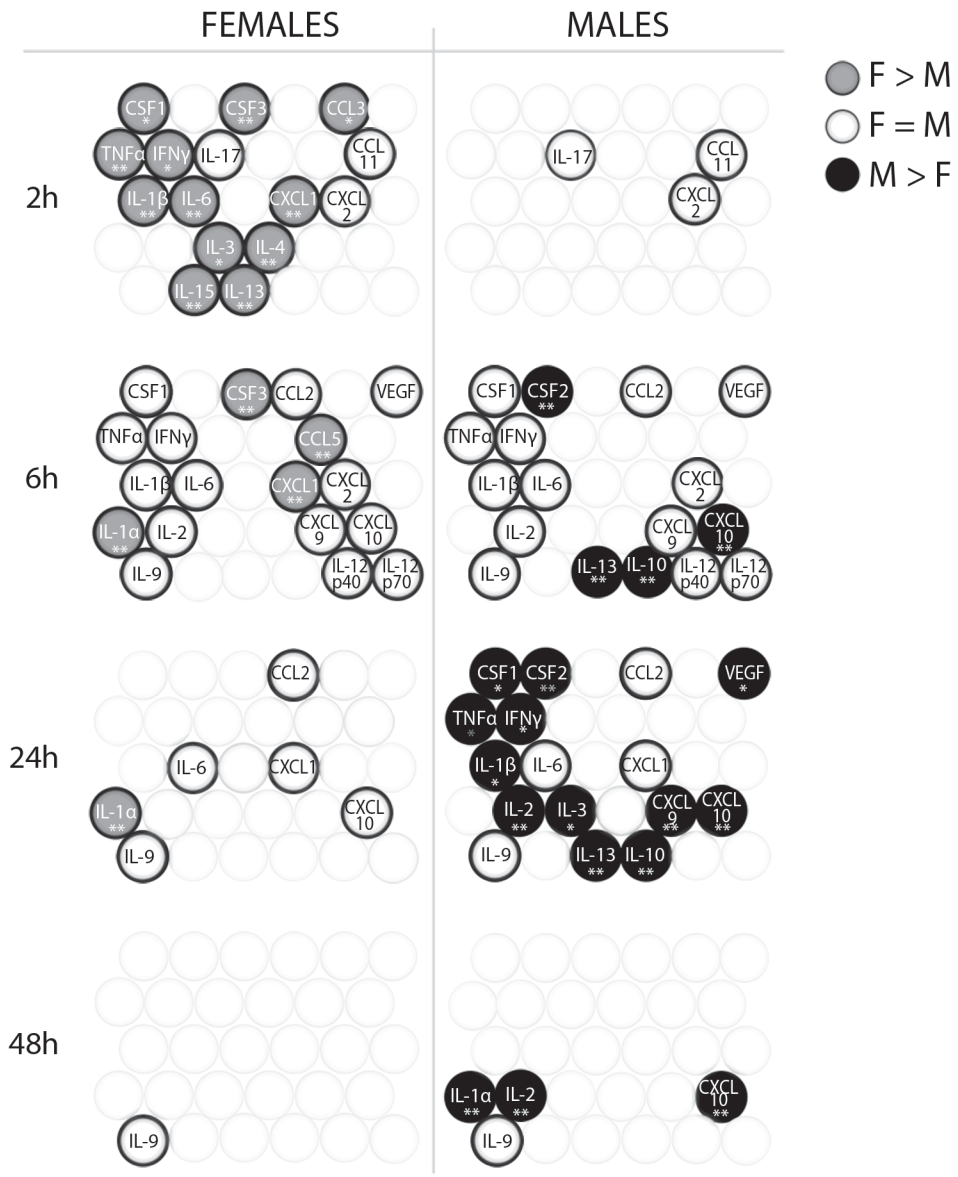

Figure 1. Sex-specific patterns and time course of cytokine activation in the hippocampus. Black circles: males > females; Grey circles: females > males; White circles: activated in both sexes. *$p < 0.05$; **$p < 0.01$ male *vs* female post hoc tests.

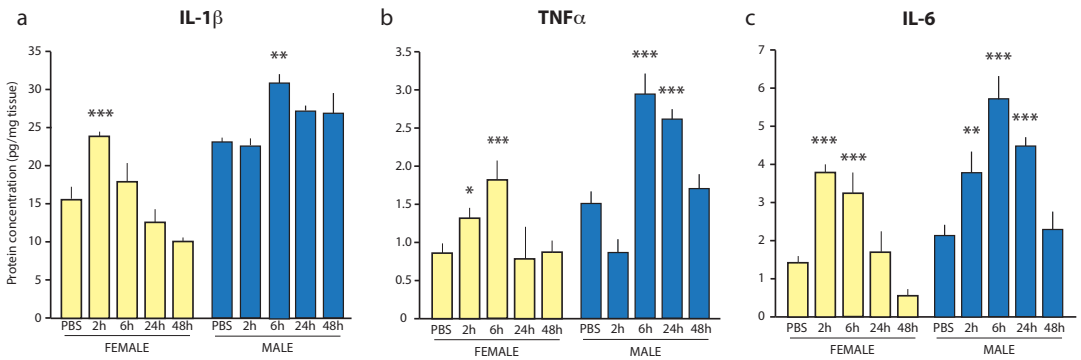

Figure 2. IL-1β, TNFα, and IL-6 are increased in the hippocampus of males and females after systemic LPS injection. (a) IL-1β is increased 6 hours after LPS injection in males and 2 hours after LPS in females. (b) TNFα is elevated at 6 hours after LPS in both male and female hippocampus. In males, this activation persists for at least 24 hours. (c) IL-6 is increased 6-24 hours after LPS in males, and earlier in females (2-6 hours). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ all *cf* PBS.

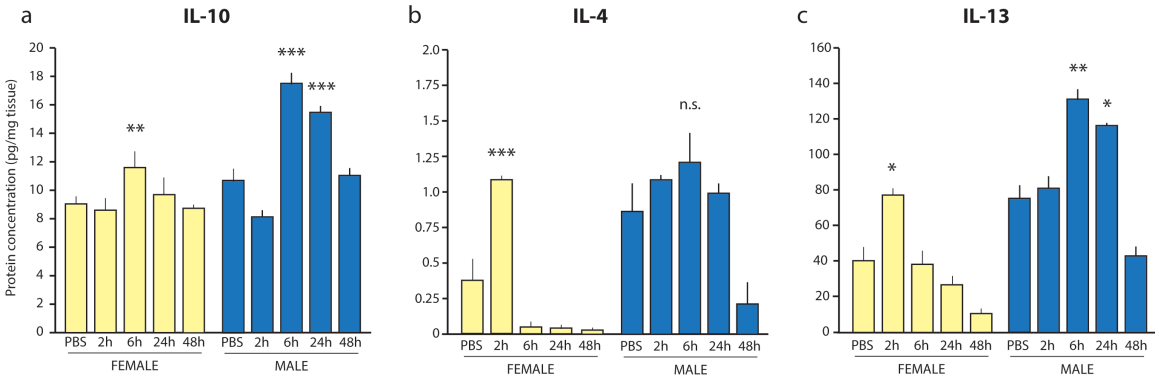

Figure 3. Regulatory cytokines IL-10, IL-4, and IL-13 are differentially activated in males and females. (a) IL-10 is elevated only in males 6-24 hours after LPS injection; (b) IL-4 is elevated only in females 2 hours after LPS injection; (c) IL-13 is activated in females at 2 hours, and in males 6-24 hours after LPS. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ all cf PBS.

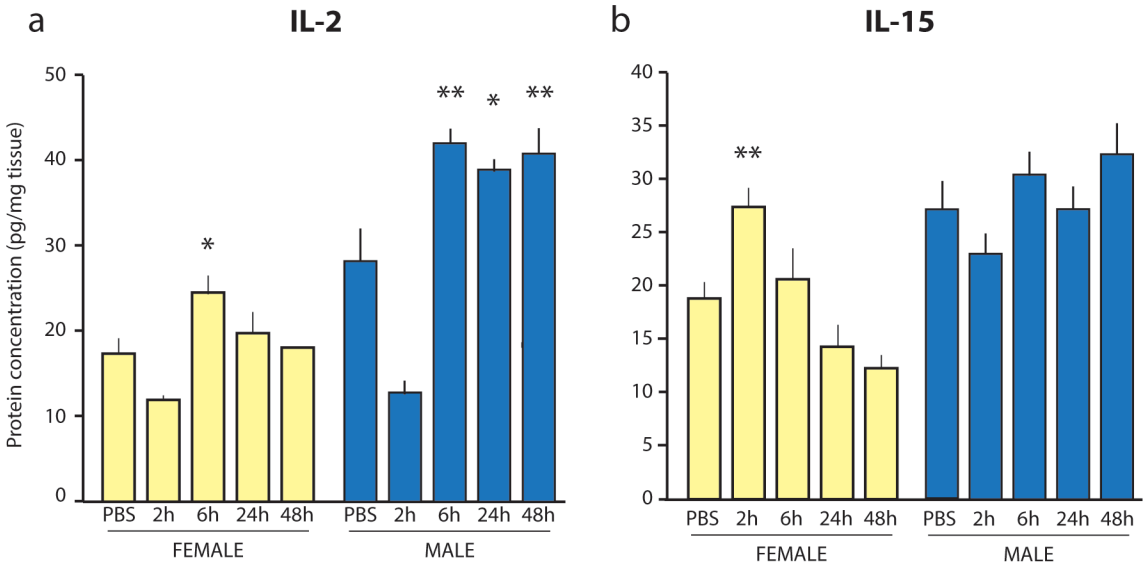

Figure 4. IL-2 and IL-15 are increased only in females. Both (a) IL-2 is elevated at 6 hours and (b) IL-15 is elevated 2 hours after LPS in females, but not males. *$p < 0.05$, **$p < 0.01$ all cf PBS.

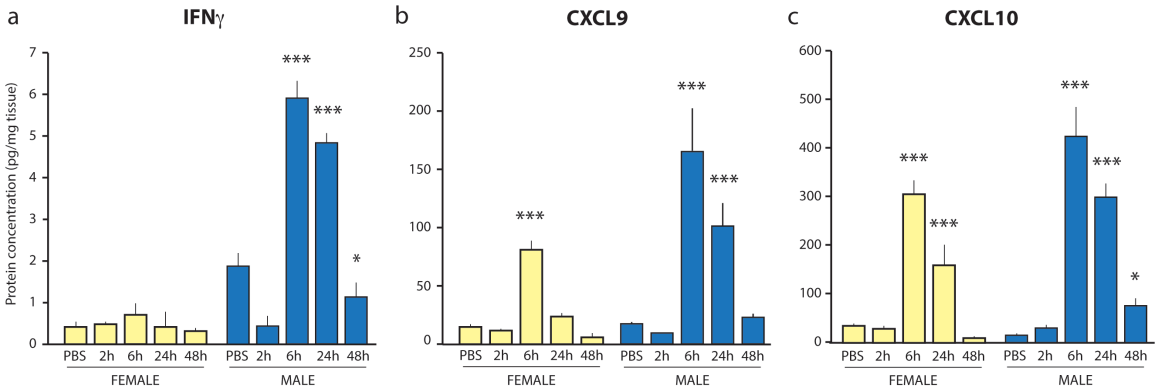

Figure 5. IFNγ, CXCL9, and CXCL10. (a) IFNγ is elevated only in males 6-24 hours after systemic LPS injection. (b) CXCL9 is elevated in both males and females 6 hours after LPS, and persists for at least 24 hours only in males. (c) CXCL10 is increased in both males and females 6-24 hours after LPS. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ all *cf* PBS.

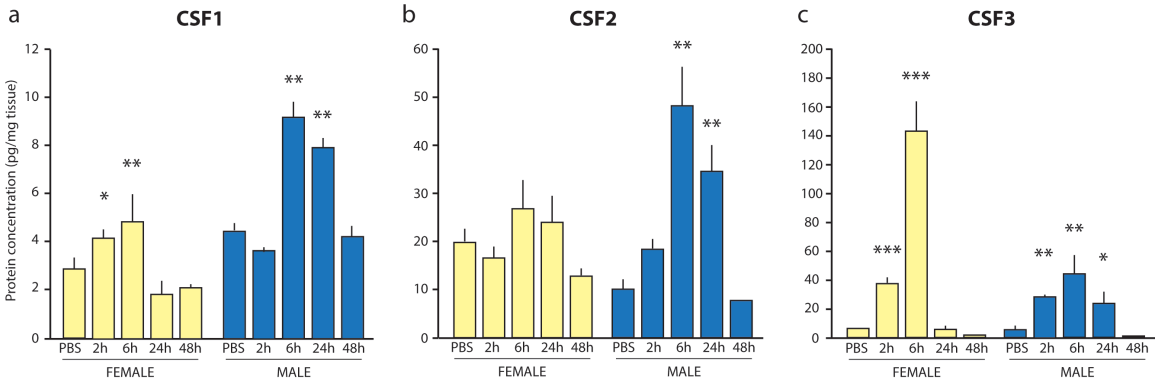

Figure 6. Colony Stimulating Factor Family. (a) CSF1 and (b) CSF2 are elevated in males but not females, 6-24 hours after LPS injection. (c) CSF3 is significantly elevated in females 2-6 hours after LPS, and to a lesser degree in males at the 6 hour timepoint. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ all *cf* PBS.

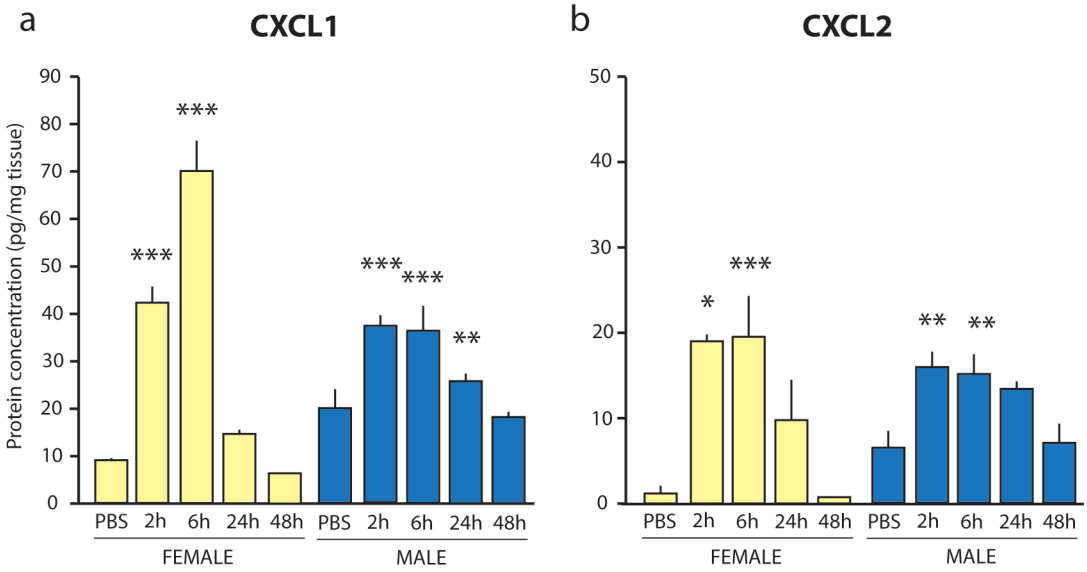

Figure 7. The CXCR2 ligands CXCL1 and CXCL2 (Gro Family)**.** (a) CXCL1 is increased in both males and females 2-6 hours after LPS. In females, this elevation is significantly stronger 6 hours post LPS compared with males. (b) CXCL2 is similarly elevated in both males and females 2-6 hours after LPS. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ all *cf* PBS.

CYTOKINE	MAIN EFFECT: TIME			INTERACTION: SEX x TIME		
Serum cytokines differentially activated in males and females						
IL-1β	***	$F_{(5,66)} = 88.05$	$\eta^2_p = 0.87$	**	$F_{(5,66)} = 4.25$	$\eta^2_p = 0.24$
CCL3 (MIP1α)	***	$F_{(5,66)} = 124.39$	$\eta^2_p = 0.90$	**	$F_{(5,66)} = 3.39$	$\eta^2_p = 0.20$
CCL4 (MIP1β)	***	$F_{(5,66)} = 25.10$	$\eta^2_p = 0.66$	***	$F_{(5,66)} = 5.67$	$\eta^2_p = 0.30$
CCL5 (RANTES)	***	$F_{(5,66)} = 32.56$	$\eta^2_p = 0.71$	***	$F_{(5,66)} = 10.08$	$\eta^2_p = 0.50$
CCL11 (Eotaxin)	**	$F_{(5,66)} = 4.78$	$\eta^2_p = 0.27$	*	$F_{(5,66)} = 2.87$	$\eta^2_p = 0.18$
CXCL2 (MIP2)	***	$F_{(5,66)} = 214.58$	$\eta^2_p = 0.94$	**	$F_{(5,66)} = 3.70$	$\eta^2_p = 0.22$
CXCL10 (IP-10)	***	$F_{(5,66)} = 64.82$	$\eta^2_p = 0.83$	**	$F_{(5,66)} = 4.27$	$\eta^2_p = 0.24$
Serum cytokines activated in both sexes						
IL-1α	*	$F_{(5,66)} = 2.53$	$\eta^2_p = 0.16$	n.s.		
TNFα	***	$F_{(5,66)} = 230.87$	$\eta^2_p = 0.95$	n.s.		
IL-6	***	$F_{(5,66)} = 338.02$	$\eta^2_p = 0.96$	n.s.		
IL-10	***	$F_{(5,66)} = 71.24$	$\eta^2_p = 0.84$	n.s.		
IL-4	***	$F_{(5,66)} = 6.39$	$\eta^2_p = 0.33$	n.s.		
IL-13	***	$F_{(5,66)} = 23.28$	$\eta^2_p = 0.64$	n.s.		
IL-15	***	$F_{(5,66)} = 16.26$	$\eta^2_p = 0.55$	n.s.		
IFNγ	*	$F_{(5,66)} = 2.53$	$\eta^2_p = 0.16$	n.s.		
CXCL9 (MIG)	***	$F_{(5,66)} = 69.68$	$\eta^2_p = 0.84$	n.s.		
CSF2 (GM-CSF)	***	$F_{(5,66)} = 50.16$	$\eta^2_p = 0.79$	n.s.		
CXCL1 (KC)	***	$F_{(5,66)} = 121.07$	$\eta^2_p = 0.90$	n.s.		
CCL2 (MCP1)	***	$F_{(5,66)} = 59.97$	$\eta^2_p = 0.82$	n.s.		
IL-3	***	$F_{(5,66)} = 6.63$	$\eta^2_p = 0.33$	n.s.		
IL-5	***	$F_{(5,66)} = 5.77$	$\eta^2_p = 0.30$	n.s.		
IL-7	***	$F_{(5,66)} = 21.25$	$\eta^2_p = 0.62$	n.s.		
IL-12p40	***	$F_{(5,66)} = 63.82$	$\eta^2_p = 0.83$	n.s.		
IL-12p70	***	$F_{(5,66)} = 12.52$	$\eta^2_p = 0.49$	n.s.		
IL-17	***	$F_{(5,66)} = 18.09$	$\eta^2_p = 0.58$	n.s.		
LIF	***	$F_{(5,66)} = 66.31$	$\eta^2_p = 0.83$	n.s.		

Table 2. Serum cytokines: Statistical analysis. Overall multivariate ANOVA results for the Main effect of TIME and the interaction effect (SEX x TIME).*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

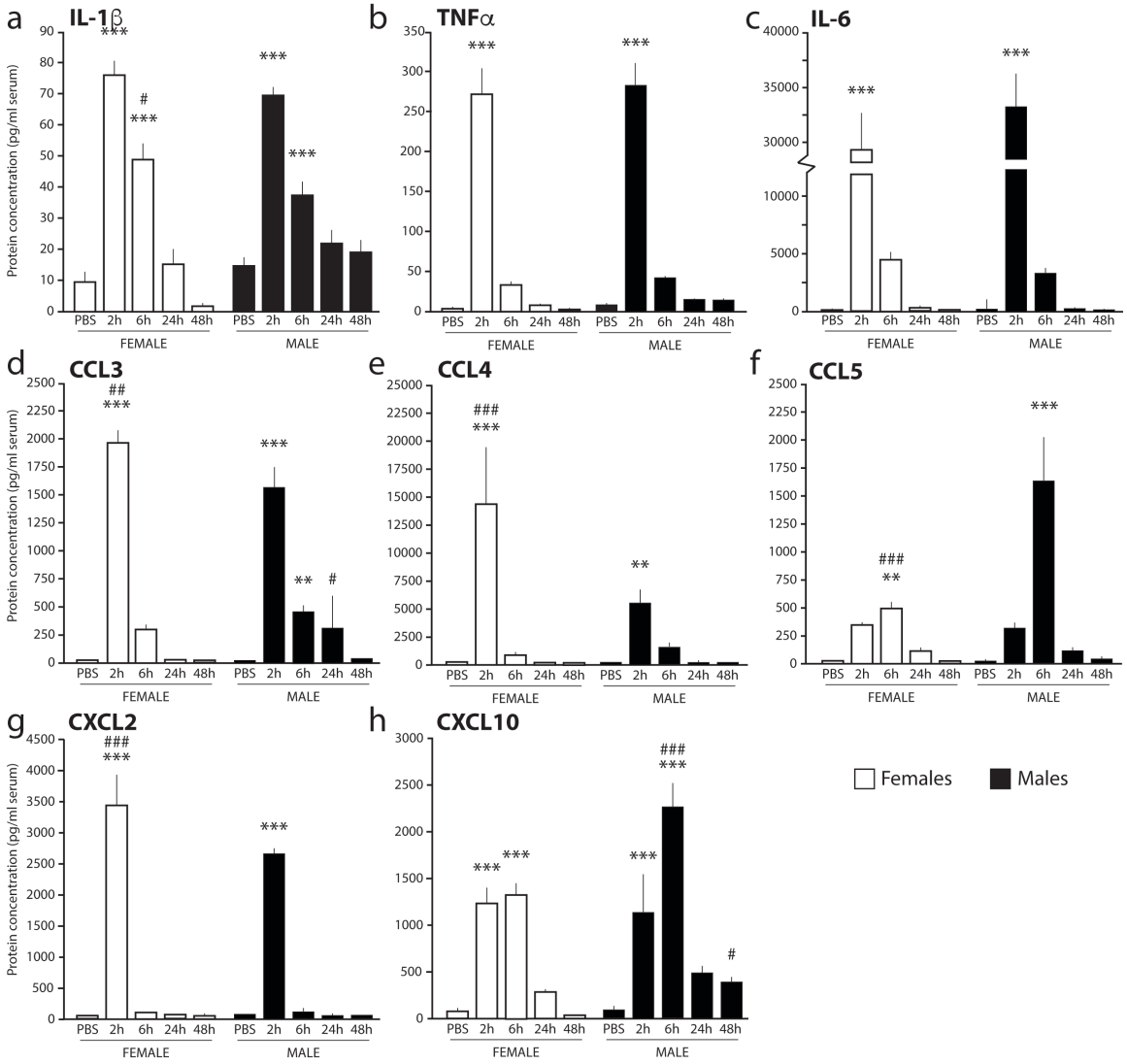

Figure 8. Serum cytokines in males and females after systemic LPS injection. (a) IL-1β is increased 2 and 6 hours after LPS in females and males. (b) TNFα is elevated 2 hours after LPS in both sexes. (c) IL-6 is increased 2 hours after LPS in males and in females. (d) CXCL2 is increased 2 hours after LPS in both sexes, and more strongly in females. (e) CXCL10 is increased 2-6 hours after LPS in both sexes, and more strongly in males. (f) CCL3 is elevted more strongly in females 2 hours after LPS, and more persistently in males. (g) CCL4 is more strongly elevated in females 2 hours after LPS. (h) CCL5 is elevated more strongly in males 2 hours after LPS. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ all cf PBS; #$p < 0.05$, ### $p < 0.001$, male vs female .