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Interleukin-6 Induced by Social Stress Promotes a Unique Transcriptional Signature in the Monocytes That Facilitate Anxiety

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ABSTRACT

BACKGROUND: Interleukin-6 (IL-6) is elevated in circulation with chronic stress and may contribute to neurobehavioral complications. We have reported that repeated social defeat stress in mice caused recruitment of proinflammatory monocytes to the brain and triggered the onset of anxiety-like behavior. Therefore, the purpose of this study was to determine the role of IL-6 signaling in the peripheral immune response, neuroinflammation, and anxiety following stress.

METHODS: Wild-type and IL-6 knockout mice were subjected to repeated social defeat, and immune and behavioral parameters were determined 14 hours later.

RESULTS: Although monocyte release and recruitment to the brain during stress were maintained in the IL-6 knockout mice, anxiety and social avoidance were prevented. NanoString analysis of fluorescence-activated cell–sorted blood monocytes ($CD11b^{+}/Ly6C^{hi}$) and brain monocytes ($CD11b^{+}/CD45^{hi}$) revealed a unique pattern of immune-related gene expression that was dependent on stress and IL-6. For instance, blood monocytes after stress had a transcriptional signature and immune profile consistent with priming, which was attenuated in monocytes from IL-6 knockout stress mice. Moreover, the monocytes recruited to the brain and associated with the development of anxiety had a transcriptional signature (enhanced *IL-1β, CD14, Mmp9, Myd88, Ager,* and *Stat3*) that was dependent on IL-6.

CONCLUSIONS: Here, we show the effects of IL-6 on the transcriptional signature of monocytes in circulation and brain after stress. Overall, robust increases in IL-6 after stress induced a primed profile in monocytes that were recruited to the brain and propagated IL-1-mediated inflammation and anxiety.

Keywords: Anxiety, IL-1β, IL-6, Monocytes, Social avoidance, Stress

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The role of inflammatory cytokines in the development and progression of psychiatric illnesses is significant because approximately 20% of the patients fail to respond to treatment and 60% fail to attain the desired response (1,2). Furthermore, patients with mood disorders nonresponsive to selective serotonin reuptake inhibitors showed significantly higher levels of peripheral interleukin-6 (IL-6) (3,4). Similarly, patients with depression nonresponsive to antidepressants with high levels of C-reactive protein showed improved symptom outcomes following anti-inflammatory intervention (5). High levels of C-reactive protein and IL-6 were also reported in individuals with anxiety disorders (6,7). IL-6 is a consistently elevated biomarker of chronic stress in long-term caregivers (8), early life adversity (9), and depressed individuals who attempted suicide (10). IL-6 is a pleiotropic cytokine produced by immune and nonimmune cells, including T cells, neutrophils, adipocytes, hepatocytes, and myocytes. IL-6 signaling is complex in that cells normally unresponsive to IL-6 may become responsive via binding of soluble receptors (11). Peripheral blood cells isolated from stressed individuals show increased production of spontaneous IL-6 and in response to immune challenge (12,13). While myriad studies detect higher circulating IL-6 levels in mood disorders, the functional role of IL-6 in mediating neurobehavioral complications is unclear.

In parallel to clinical studies, rodents exposed to stressors (e.g., restraint, footshock, social defeat stress) have increased circulating IL-6 (14–17). Increase in plasma IL-6 is regulated by the hypothalamic-pituitary-adrenal axis. For instance, interfering with hypothalamic-pituitary-adrenal activation by adrenalectomy or metyrapone (corticosterone synthesis inhibitor) prevented plasma IL-6 production during stress (15,17). Rodent studies have provided some insight into the functional role of IL-6 in behavioral dysregulation. For example, chronic stress and overexpression of central IL-6 triggered depressive-like behavior in rats—an effect unaltered by selective serotonin reuptake inhibitors but prevented by treatment with IL-6 antibodies (18). Notably, IL-6 knockout ($IL\text{-}6^{KO}$) mice were resilient to learned helplessness behavior in response to uncontrollable footshocks, forced swim, and tail suspension (19). Furthermore, mice with social avoidance (i.e., susceptible) following

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exposure to social defeat stress had higher IL-6 levels compared with those that did not develop social avoidance (i.e., resilient), and this effect was prevented with IL-6 antibody treatment (16). Bone marrow transplantation from susceptible mice induced social avoidance in naïve mice after a single subthreshold exposure to stress (16). A recent study indicated that peripheral IL-6 may directly enter the brain via increased blood-brain barrier permeability and promote social avoidance following social defeat stress (20). Taken together, clinical and experimental findings highlight a key role of IL-6 in neurobehavioral deficits following stress.

We have reported that repeated social defeat (RSD) in mice promoted the accumulation of peripheral monocytes in the brain that augmented IL-1β signaling and caused prolonged anxiety-like behavior (21,22). RSD also induced a robust increase in plasma IL-6 (17,23). Interventions with noradrenergic receptor antagonist or gamma-aminobutyric acidergic agonists prevented RSD-induced IL-6 increase, monocyte recruitment to the brain, and anxiety-like behavior following RSD (24,25). Therefore, we aimed to determine the role of IL-6 signaling in the peripheral immune response, neuroinflammation, and anxiety following RSD. Here, we show novel data that IL-6 production during RSD primes peripheral monocytes that traffic to the brain and augment IL-1β signaling that is associated with anxiety-like behavior.

METHODS AND MATERIALS

Mice

Wild-type (WT) male C57BL/6 mice (6–8 weeks old) and CD-1 mice (12 months old) were purchased from Charles River Laboratories (Wilmington, MA). Breeding triads of IL-6^{KO} mice on the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). Additional information is provided in the Supplement. All procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Repeated Social Defeat

Age-matched WT and transgenic mice (7–8 weeks old) were exposed to RSD as previously described (21) and outlined in the Supplement. In brief, an aggressive CD-1 mouse was introduced into the cage of an established cohort of three resident C57BL/6 mice 2 hours daily for 6 consecutive days, and resident mice were monitored for submissive behaviors. At the end of the 2-hour period, the aggressive mice were placed back into their cages until the next day when the protocol was repeated.

Behavioral Analyses

Fourteen hours after the last cycle of stress, mice were tested for anxiety-like behavior in the open field and social avoidance as described in the Supplement.

Tissue Collection for Enzyme-Linked Immunosorbent Assay, Flow Cytometry, and Real-Time Quantitative Polymerase Chain Reaction

Bone marrow, blood, and brain samples were collected following carbon dioxide asphyxiation, 14 hours after the last cycle of stress, and processed for flow cytometry and enzyme-linked immunosorbent assay as described in the Supplement. From a separate experiment, brain $CD11b^+$ cells were collected immediately after the last cycle of stress for real-time quantitative polymerase chain reaction (see Supplement).

Immunohistochemistry

Fourteen hours after the last cycle of stress, mice were transcardially perfused and fixed with paraformaldehyde. Brain samples were processed, and Iba-1 immunofluorescence was performed as described in the Supplement.

NanoString Gene Expression Analysis

Blood monocytes ($CD11b^+$/$Ly6C^{hi}$) and brain monocytes ($CD11b^+$/$CD45^{hi}$) were collected via fluorescence-activated cell sorting 14 hours after the last cycle of stress. RNA copy number was determined for 279 genes using the nCounter Mouse Inflammation v2 Panel Plus (NanoString Technologies, Seattle, WA). Geometric means of positive controls were used to normalize the housekeeping genes, which were then used to normalize the samples. All normalization and data analysis was performed using nSolver Analysis Software 4.0 (NanoString Technologies). The cutoff for significance was set to $p < .05$ for differentially expressed genes. Genes differentially expressed following RSD in WT and IL-6^{KO} mice were used for Ingenuity Pathway Analysis (IPA) of upstream regulators (QIAGEN Silicon Valley, Redwood City, CA).

Ex Vivo Lipopolysaccharide Stimulation of Peripheral Blood Mononuclear Cells

Blood samples were collected 14 hours after the last cycle of stress. Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll gradient and cultured for 18 hours with lipopolysaccharide (LPS).

Statistical Analyses

Data are presented as the average ± SEM for each treatment group. Data were analyzed using two-way (stress × intervention) analysis of variance using SPSS Statistics 25 (IBM Corp., Armonk, NY). In the case of main effect of condition (control vs. stress) or intervention (WT vs. IL-6^{KO}) or an interaction, differences between group means were evaluated by an *F*-protected *t* test. Post hoc analysis results are depicted graphically in the figures.

RESULTS

Stress-Induced Anxiety and Social Avoidance Were Prevented in IL-6-Deficient Mice

IL-6 is implicated in stress-induced neuropsychiatric deficits, but the functional role of IL-6 is unclear. Here, we aimed to determine the role of IL-6 in the monocyte-mediated response to stress and the development of anxiety. First, anxiety-like behavior and social avoidance were determined in WT and IL-6^{KO} mice 14 hours after the last cycle of RSD (stress). Representative heat maps of activity for each experimental group are shown in the open field (Figure 1A). There was a trend for a main effect of stress ($p = .06$) and a significant

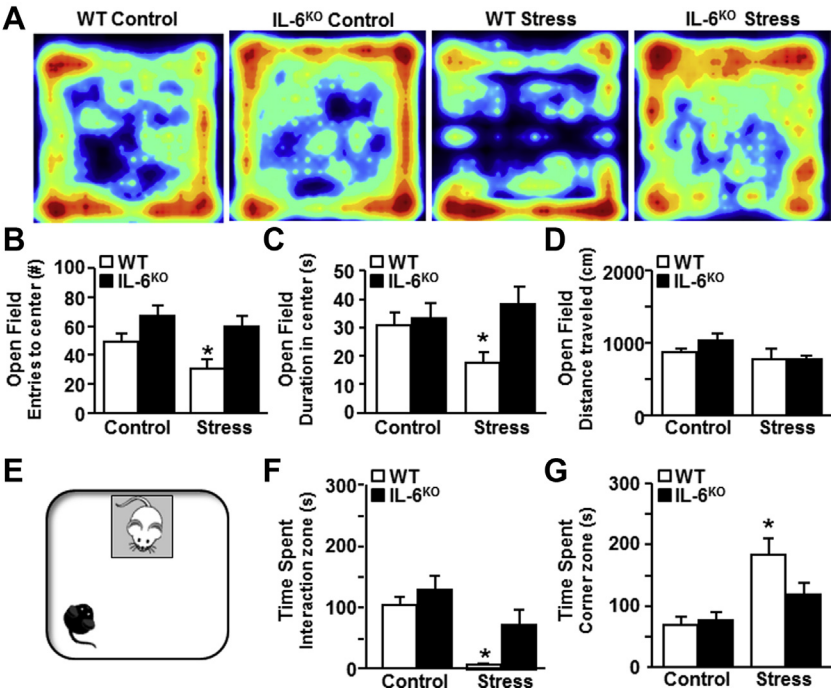

Figure 1. Stress-induced anxiety and social avoidance were prevented in interleukin-6 (IL-6)-deficient mice. Male wild-type (WT) and IL-6 knockout (IL-6^{KO}) C57BL/6 mice were subjected to six repeated cycles of social defeat (stress) or left undisturbed as control mice. Anxiety-like (n = 10) and social avoidance (n = 8–10) behaviors were determined 14 hours later. **(A)** Representative heat maps of activity in the open field. **(B–D)** Number of entries into the center (stress, $F_{1,40} = 3.7$, $p = .06$; genotype, $F_{1,40} = 11.8$, $p < .01$) **(B)**, duration in center (genotype, $F_{1,40} = 4.9$, $p < .05$; stress × genotype, $F_{1,40} = 3.0$, $p = .09$) **(C)**, and distance traveled in the open field test **(D)**. Next, social avoidance behavior was determined in the same mice. **(E)** Schematic diagram of the social avoidance test showing the social trial. **(F)** Time spent in the interaction zone during social trial (stress, $F_{1,34} = 12.3$, $p < .01$; genotype, $F_{1,34} = 4.3$, $p < .05$). **(G)** Time spent in the corner zone during social trial (stress, $F_{1,33} = 19.5$, $p < .0001$; stress × genotype, $F_{1,33} = 4.1$, $p = .05$). Bars represent mean ± SEM. Bars with asterisk (*) are significantly different from the control mice (post hoc analysis, $p < .05$).

effect of genotype ($p < .01$) on entries into the center of the open field (Figure 1B). Post hoc analysis confirmed that WT-stress mice had fewer entries into the center of the open field compared with all other groups ($p < .05$). Duration in the center of the open field after stress (Figure 1C) showed effects of genotype ($p < .05$) with a trend toward interaction (stress × genotype, $p = .08$). Again, post hoc analysis confirmed that WT-stress mice spent the least amount of time in the center compared with all other groups ($p < .05$). Locomotor activity was unaffected by stress or genotype (Figure 1D).

Social avoidance behavior was also determined after stress. During the empty trial of the social avoidance test, neither stress nor genotype altered the amount of time spent in the interaction and corner zones (data not shown). During the social trial where a CD-1 mouse was present in the cage (Figure 1E), both time spent in the interaction zone (Figure 1F) (stress, $p < .01$; genotype, $p < .05$) and time spent in the corner zone (Figure 1G) (stress, $p < .0001$; interaction, $p = .05$) were affected by stress and genotype. Post hoc analysis confirmed that WT-stress mice spent the least amount of time in the interaction zone and the most amount of time in the corner zone compared with all other groups ($p < .05$ for each). Collectively, stress-induced anxiety and social avoidance were prevented in the IL-6^{KO} mice.

Stress-Induced Myeloid Cell Production and Release Into Circulation Were Maintained in the IL-6-Deficient Mice

Next, the effect of IL-6 deficiency on the production and release of monocytes and granulocytes after stress was assessed. As a control, IL-6 levels were also determined in the plasma after stress. As expected, stress increased plasma IL-6 (Figure 2A) ($p < .05$), and this increase was prevented in the IL-6^{KO} mice (Figure 2A) (stress × genotype, $p < .01$). Stress also increased the percentage of monocytes ($CD11b^{+}/Ly6C^{hi}$) and granulocytes ($CD11b^{+}/Ly6G^{+}$) in the bone marrow (Figure 2B–D) ($p < .0001$) and circulation (Figure 2E–G) ($p < .0001$). These immune responses to stress were independent of genotype. Overall, stress induced an increase in the production and release of myeloid cells in both WT and IL-6^{KO} mice.

IL-6 Deficiency Attenuated the Stress-Induced Brain IL-1β Expression but Did Not Affect Stress-Induced Microglia Activation and Monocyte Recruitment to the Brain

Inflammatory monocytes are recruited to the brain by activated microglia (chemokine-dependent) and provide IL-1β signal to the brain endothelial cells. This immune-to-brain signaling mediated by monocyte-derived IL-1β is critical to the induction of anxiety-like behavior (21). Therefore, microglial Iba-1 expression was assessed after stress in WT and IL-6^{KO} mice to determine microglial structural activation, which is associated with chemokine induction after stress. Stress increased Iba-1 proportional area in the prelimbic cortex (Figure 3A, B) ($p < .001$), CA3 (Figure 3C) ($p < .01$), and dentate gyrus (Figure 3D) ($p < .01$). For example, microglia in the stressed brain had larger elongated cell bodies and thicker processes compared with control mice (Figure 3A). These morphological changes marked by increased Iba-1 are associated with an activated state of microglia (21,26). Increased Iba-1 expression occurred in both WT and IL-6^{KO} mice. Microglia numbers did not significantly differ among the groups (Figure 3E). Thus,

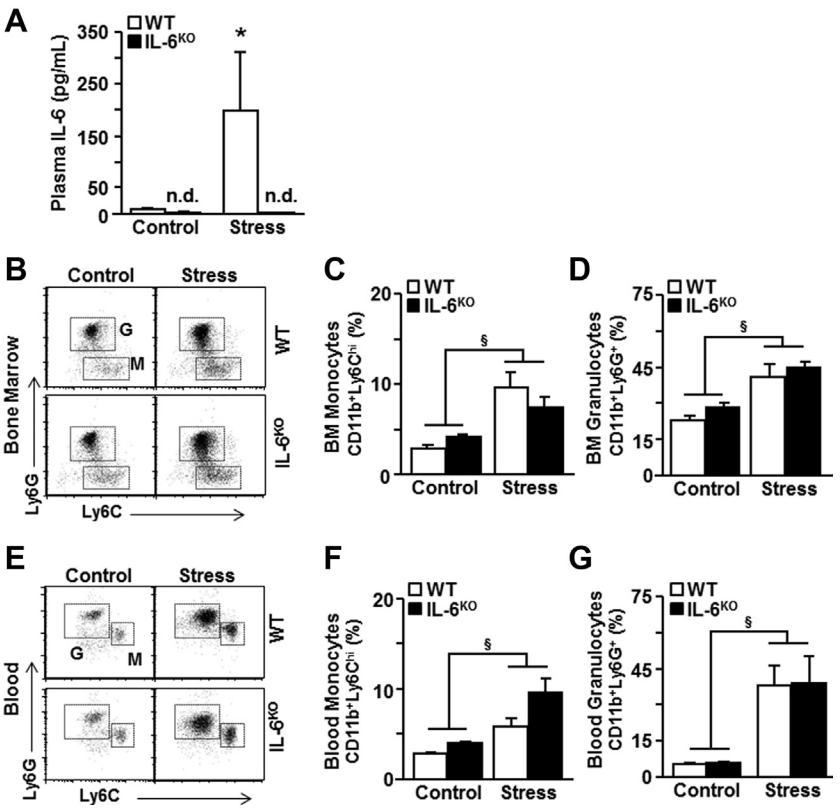

Figure 2. Stress-induced myeloid cell production and release into circulation were maintained in interleukin-6 (IL-6)-deficient mice. Male wild-type (WT) and IL-6 knockout (IL-6^{KO}) C57BL/6 mice were subjected to six repeated cycles of social defeat (stress) or left undisturbed as control mice. Several immune parameters were determined 14 hours later (n = 4–5). **(A)** IL-6 concentration in the plasma (stress, $F_{1,19} = 8.8$, $p < .01$; genotype, $F_{1,19} = 9.8$, $p < .01$; stress × genotype, $F_{1,19} = 8.9$, $p < .01$). **(B)** Representative bivariate dot plots of Ly6G and Ly6C labeling of $CD11b^+$ cells in the bone marrow (BM). **(C)** Percentage of monocytes ($CD11b^+/Ly6C^{hi}$) in the BM (stress, $F_{1,19} = 29.3$, $p < .0001$). **(D)** Percentage of granulocytes ($CD11b^+/Ly6G^+$) in the BM (stress, $F_{1,18} = 20.8$, $p < .0001$). **(E)** Representative bivariate dot plots of Ly6G and Ly6C labeling of $CD11b^+$ cells in the blood. **(F)** Percentage of monocytes ($CD11b^+/Ly6C^{hi}$) in the blood (stress, $F_{1,10} = 50.7$, $p < .0001$; stress × genotype, $F_{1,10} = 5.4$, $p = .05$). **(G)** Percentage of granulocytes ($CD11b^+/Ly6G^+$) in the blood (stress, $F_{1,11} = 36.3$, $p < .0001$). Bars represent mean ± SEM. Bars with section symbol (§) indicate significant main effect of stress ($p < .05$). Bars with asterisk (*) are significantly different from the control mice (post hoc analysis, $p < .05$).

microglial morphological activation occurred following stress in an IL-6-independent manner.

Next, monocyte accumulation in the brain and chemokine and IL-1β expression in microglia/monocytes were determined in WT and IL-6^{KO} mice after stress. Stress increased the presence of $CD11b^+/CD45^{hi}$ cells in the brain (Figure 3F, G) ($p < .001$) in a genotype-independent manner. Next, chemokine and cytokine expression was analyzed immediately after stress. This immediate time point was chosen because CCL2 and CCL7 levels decline at later time points. Real-time quantitative polymerase chain reaction of enriched $CD11b^+$ cells (microglia/monocytes) showed increased CCL2 and CCL7 levels after stress (Figure 3H, I) ($p < .01$ for each). Again, these increases were independent of genotype. Stress increased IL-1β messenger RNA expression in the enriched $CD11b^+$ cells, and this effect tended to be genotype dependent (Figure 3J) (stress, $p < .01$; interaction, $p = .08$). Post hoc analysis confirmed that WT-stress mice had the highest level of IL-1β compared with all other groups ($p < .05$). Collectively, stress caused microglia activation and monocyte recruitment to the brain independent of genotype, but the corresponding induction of IL-1β was prevented in IL-6^{KO} mice.

A Primed Immune Signature of Blood Monocytes After Stress Was Dependent on IL-6

We have shown previously that circulating monocytes recruited to the brain during stress relay an IL-1β signal to the brain, augmenting the neuroinflammatory profile and subsequently inducing anxiety-like behavior in mice (21). Here, we show that IL-6 plays a role in two key responses to stress: behavior and brain IL-1β expression. Thus, the absence of IL-6 signaling in the IL-6^{KO} mice may alter the activation profile of the monocytes released into circulation during stress. To address this hypothesis, monocytes ($CD11b^+/Ly6C^{hi}$) were isolated from the blood 14 hours after stress, and RNA copy number of 279 immune-related genes was determined using nCounter NanoString analysis (Figure 4A).

The volcano plot ($-\log_{10}$ [p value] vs. fold change) in Figure 4B shows genes differentially expressed in blood monocytes between the WT-stress and WT-control mice. Genes increased and decreased in monocytes after stress ($p < .05$) are labeled in red and blue, respectively. Figure 4C shows genes differentially expressed between IL-6^{KO}-stress and WT-stress mice. Figure 4D shows genes differentially expressed between IL-6^{KO}-stress and IL-6^{KO}-control mice. The Venn diagram (Figure 4E) shows that there was a total of 37 genes (black + gray) significantly altered in blood monocytes after stress. Within this group, 30 genes were different between WT-stress and WT-control mice but not different between IL-6^{KO}-stress and WT-stress mice (black), and seven of these stress-altered genes were reversed in the IL-6^{KO}-stress mice (gray; different between IL-6^{KO}-stress and WT-stress mice). Furthermore, there were 12 genes (white) unaltered between the WT-stress and WT-control mice, but they were significantly altered between IL-6^{KO}-stress and

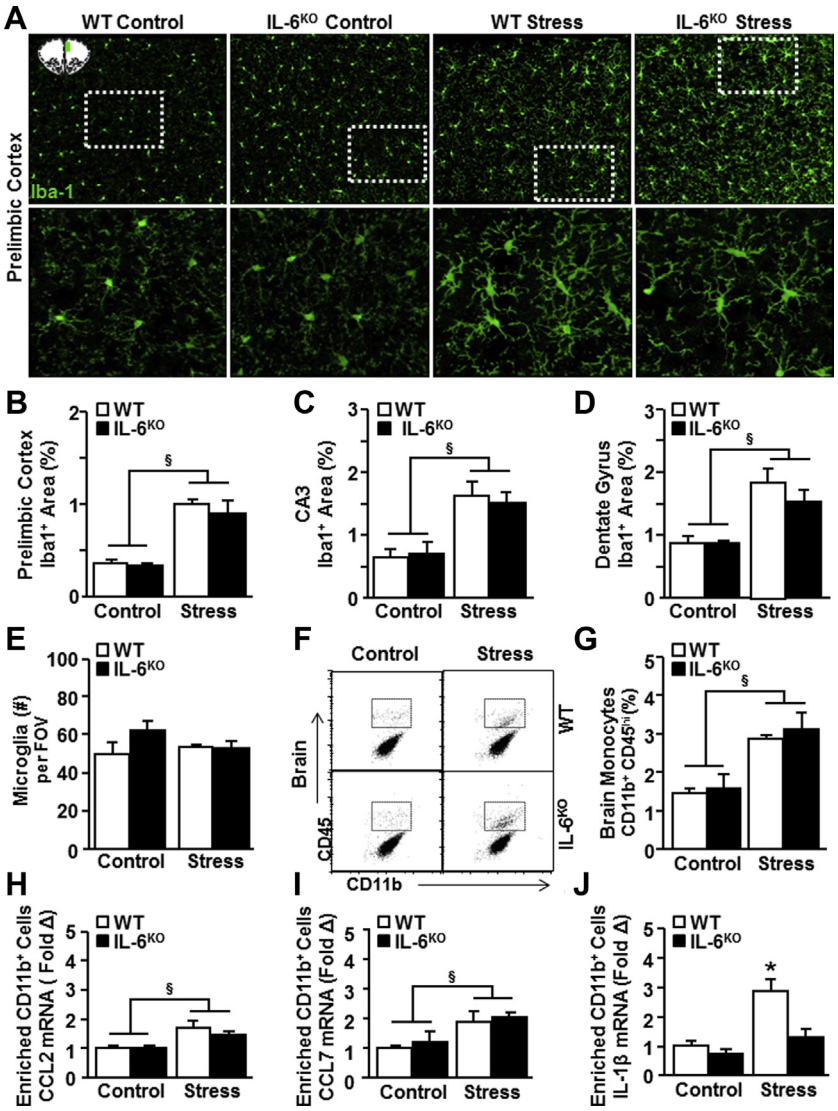

Figure 3. Interleukin-6 (IL-6) deficiency attenuated the stress-induced brain IL-1β expression but did not affect stress-induced microglia activation and monocyte recruitment to the brain. Male wild-type (WT) and IL-6 knockout (IL-6^{KO}) C57BL/6 mice were subjected to six repeated cycles of social defeat (stress) or left undisturbed as control mice. Several neuroimmune parameters were determined 14 hours later. **(A)** Representative images of Iba-1 labeling (n = 3) in the prelimbic cortex. The images were taken within the region highlighted in the schematic (top left). Dashed rectangles indicate regions of higher magnification images (bottom panels). **(B–D)** Percentage area of Iba-1 labeling in the prelimbic cortex (stress, $F_{1,13}$ = 27.7, $p < .001$) **(B)**, CA3 (stress, $F_{1,11}$ = 18.0, $p < .01$) **(C)**, and dentate gyrus (stress, $F_{1,12}$ = 23.1, $p < .01$) **(D)**. **(E)** Number of microglia (per field of view [FOV]) in the prelimbic cortex (stress × genotype, $F_{1,10}$ = 2.6, p = .14). **(F)** Representative bivariate dot plots of CD11b and CD45 labeling of Percoll-enriched cells from the brain (n = 4–5). **(G)** Percentage of monocytes (CD11b^{+}/CD45^{hi}) in the brain (stress, $F_{1,18}$ = 22.3, $p < .001$). In a separate study, mice were exposed to stress, and Percoll-enriched CD11b^{+} cells were isolated from the brain immediately after the final exposure to stress (n = 4–5). **(H–J)** Messenger RNA (mRNA) levels of CCL2 (stress, $F_{1,15}$ = 10.0, $p < .01$) **(H)**, CCL7 (stress, $F_{1,13}$ = 12.5, $p < .01$) **(I)**, and IL-1β (stress, $F_{1,17}$ = 12.5, $p < .01$; genotype, $F_{1,19}$ = 7.2, $p < .05$; stress × genotype, $F_{1,17}$ = 3.5, p = .08) **(J)** were determined. Bars represent mean ± SEM. Bars with section symbol (§) indicate significant main effect of stress ($p < .05$). Bars with asterisk (*) are significantly different from the control mice (post hoc analysis, $p < .05$).

WT-stress mice. Taken together, stress altered the expression of 37 immune genes in monocytes, seven of which were reversed in the IL-6^{KO} group.

Based on these analyses, a selected list of genes associated with myeloid signature, chemokines/cytokines, immune signaling, and glucocorticoid signaling is shown (Supplemental Figure S1). Pairwise comparisons indicated that stress increased ($p < .05$) the RNA copy number of several key inflammatory (*Ly6c, Mmp9, Cxcr2, Alox5,* and *Ccr1*) and glucocorticoid sequestration (*Fkbp5*) genes in blood monocytes. Stress also decreased ($p < .05$) the genes associated with regulation (*Cx3cr1* and *Tgfbr1*), antigen presentation (*H2-eb1*), and interferon response (*Tlr3, Mx1, Mx2, Irf3, Ifi44,* and *Stat2*). The stress-associated alterations in *Alox5, Ly6C, Cd55, Grb2, Mmp9, Tgbr1,* and *Stat2* were absent in the IL-6^{KO}-stress mice. Taken together, the primed transcriptional profile of blood monocytes after stress was attenuated in IL-6^{KO} mice.

An IL-6-Dependent Primed Phenotype of Blood Leukocytes After Stress

A primed immune profile is functionally defined as an increased cytokine response to innate immune challenge (27). To assess priming in WT and IL-6^{KO} mice after stress, PBMCs were isolated and stimulated with ex vivo LPS. IL-6 expression in PBMCs was influenced by stress × genotype × LPS (Figure 5A) ($p < .05$). Following LPS, PBMCs from WT-stress mice had the highest IL-6 levels compared with all other groups ($p < .05$). Similarly, IL-1β was also influenced by stress × genotype × LPS (Figure 5B) ($p < .01$). Compared with WT-control mice, PBMCs from WT-stress mice

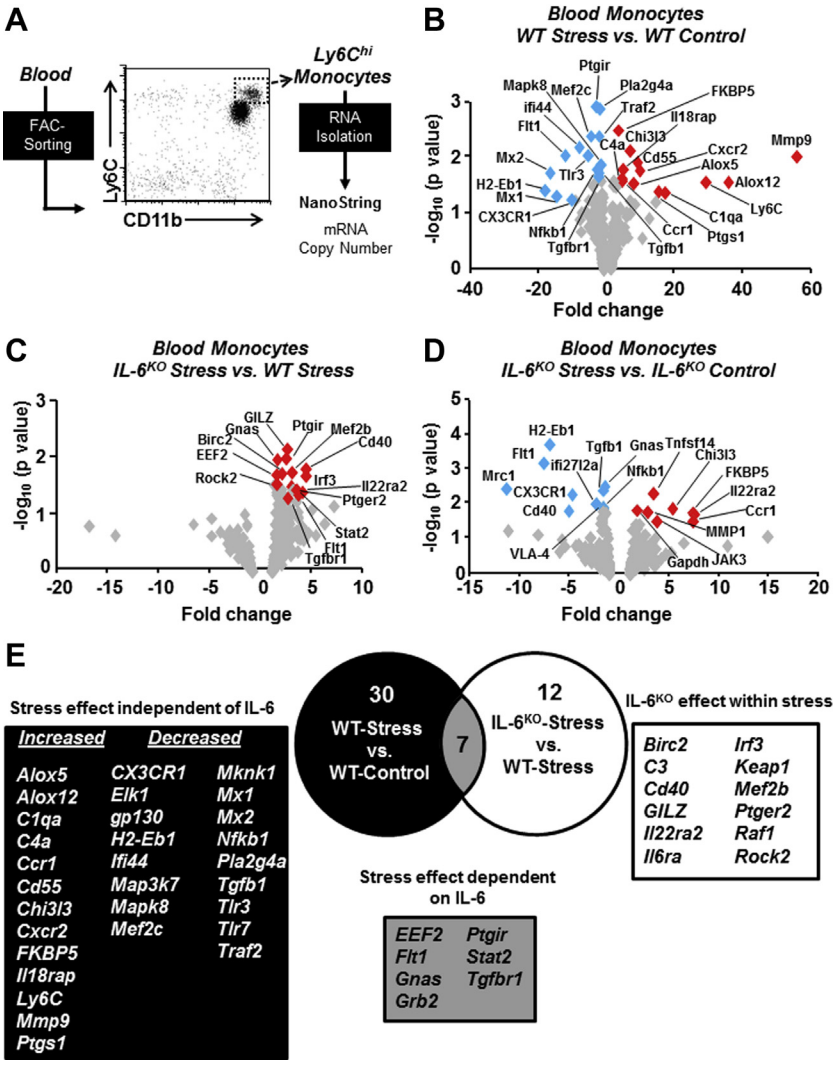

Figure 4. A primed immune signature of blood monocytes after stress was dependent on interleukin-6 (IL-6). Male wild-type (WT) and IL-6 knockout (IL-6^{KO}) C57BL/6 mice were subjected to six repeated cycles of social defeat (stress) or left undisturbed as controls. **(A)** Monocytes (CD11b^{+}/Ly$6C^{hi}$) in the blood were collected via fluorescence-activated cell (FAC) sorting (n = 4) 14 hours later. Next, RNA was isolated and messenger RNA (mRNA) copy number of 279 genes was determined using NanoString gene array. **(B–D)** Volcano plot (-$\log_{10}$ [p value] vs. fold change) of genes differentially expressed between monocytes from WT-stress and WT-control groups **(B)**, IL-6^{KO}-stress and WT-stress groups **(C)**, and IL-6^{KO}-stress and IL-6^{KO}-control groups **(D)**. Genes labeled in red (increased) and blue (decreased) were significantly different. **(E)** Venn diagram depicts genes significantly altered between WT-stress and WT-control groups but not between IL-6^{KO}-stress and WT-stress groups (black background), genes significantly altered between WT-stress and WT-control groups and between IL-6^{KO}-stress and WT-stress groups (gray background), and genes significantly altered only between the IL-6^{KO}-stress and WT-stress groups ($p < .05$ for all).

expressed higher IL-1β after LPS ($p < .05$). Notably, PBMCs from IL-6^{KO}-control mice also had high levels of IL-1β after LPS ($p < .05$). Nevertheless, IL-1β in the IL-6^{KO}-stress group was not different from that in the WT-control group after LPS. These findings revealed a primed profile characterized by enhanced IL-6 and IL-1β expression in PBMCs stimulated with LPS after stress. This primed profile was attenuated in the IL-6^{KO} mice.

The Primed and IL-1β Signature of Monocytes Recruited to the Brain During Stress Was Dependent on IL-6

Next, we assessed the transcriptional profile of monocytes (CD11b^{+}/CD45^{hi}) in the brain after stress (Figure 6A) following a similar protocol as in Figure 4A. The volcano plot (-$\log_{10}$ [p value] vs. fold change) in Figure 6B shows genes differentially expressed between WT-stress and WT-control mice. Genes increased and decreased in brain monocytes after stress ($p < .05$) are labeled in red and blue, respectively. Figure 6C shows genes differentially expressed between IL-6^{KO}-stress and WT-stress groups. Figure 6D shows genes differentially expressed between IL-6^{KO}-stress and IL-6^{KO}-control mice. IPA analysis showed that stress induced gene expression, consistent with increased signaling of IL-6, IL-8, high mobility group box 1, Janus kinase/signal transducer and activator of transcription proteins, and IL-17A pathways in brain monocytes. In contrast, stress in IL-6^{KO} mice reduced expression of genes related to IL-6, macrophage reactive oxygen species production, high mobility group box 1, Janus kinase/signal transducer and activator of transcription proteins, and IL-17A (Figure 6E). The Venn diagram (Figure 6F) shows that there was a total of 46 genes (black + gray) significantly altered in the brain monocytes after stress. Within this group, 34 genes were different between WT-stress and WT-control mice but not different between IL-6^{KO}-stress and WT-stress mice (black), and 12 of these stress-altered genes were reversed in the IL-6^{KO}-stress mice (gray; different between IL-6^{KO}-stress and WT-stress mice). Furthermore, there

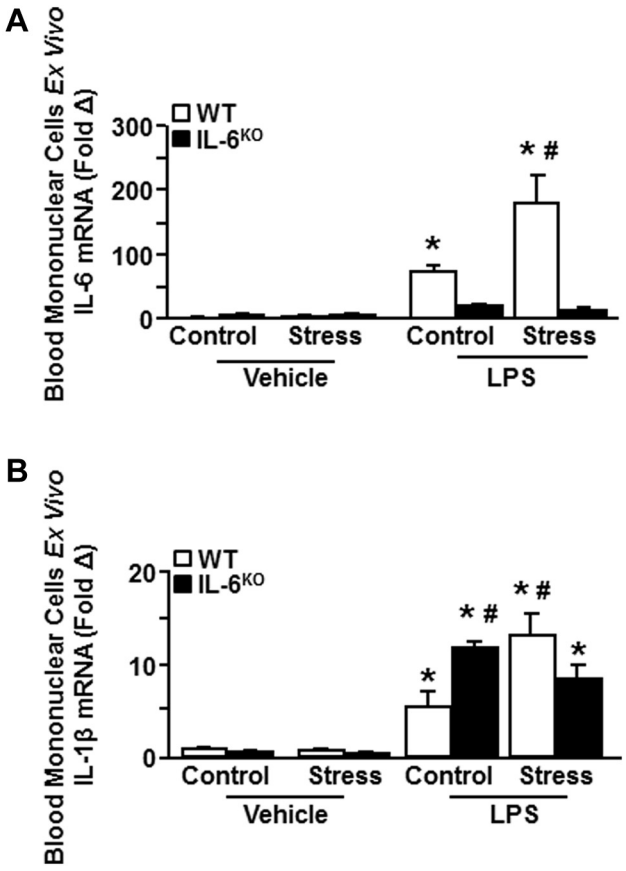

Figure 5. An interleukin-6 (IL-6)-dependent primed phenotype of blood leukocytes after stress. Male wild-type (WT) and IL-6 knockout (IL-6^{KO}) C57BL/6 mice were subjected to six repeated cycles of social defeat (stress) or left undisturbed as control mice (n = 3). Peripheral blood mononuclear cells were collected 14 hours after stress and were cultured ex vivo with lipopolysaccharide (LPS) (0.4 μg/mL) for 18 hours. **(A, B)** Messenger RNA (mRNA) levels of IL-6 expression (treatment × genotype × stress, $F_{1,22}$ = 5.9, $p < .05$) **(A)** and IL-1β expression (treatment × genotype × stress, $F_{1,47}$ = 11.2, $p < .01$) were determined. Bars represent mean ± SEM. Bars with asterisk (*) are significantly different from the vehicle control mice (post hoc analysis, $p < .05$). Bars with pound symbol (#) are significantly different from the WT LPS control mice.

were 15 genes (white) unaltered between the WT-stress and WT-control mice, but they were significantly altered between the IL-6^{KO}-stress and WT-stress mice. Taken together, stress altered the expression of 46 immune genes in brain monocytes, 12 of which were dependent on IL-6, including key inflammatory molecules (*Il-1β, Ly6C, Mmp9,* and *CD14*).

Related to these analyses, a selected list of genes associated with myeloid signature, chemokines/cytokines, immune signaling, and glucocorticoid signaling is shown (Supplemental Figure S2). The RNA signature of monocytes in the brain after stress was consistent with the profile of blood monocytes. The progression of monocytes from the blood to the brain during stress was characterized by robust induction of *Il-1β, CD14,* and *Stat3.* Overall, stress increased ($p < .05$) expression of several inflammatory-related molecules: *Ager, Alox5, CD14, Ccr1, Cxcl2, IL-1β, Mmp9, Myd88,* and *Stat3*. All of these stress-associated increases were prevented by IL-6 deficiency. Thus, monocytes recruited to the brain during stress were primed in an IL-6-dependent manner to express IL-1β.

DISCUSSION

IL-6 is a consistent cytokine biomarker in clinical and experimental reports of chronic stress and is associated with treatment-resistant mood disorders and increased morbidity and mortality (16,28). Nonetheless, there is little information on the functional effects of high IL-6 on the physiological, behavioral, and immunological response to stress. Recent studies modeling social stress in mice indicate that peripheral IL-6 can enter the brain and influence the social avoidance response (20). In addition, mice susceptible or resilient to social avoidance after stress had higher or lower levels of plasma IL-6, respectively (16). Furthermore, these behavioral effects associated with high IL-6 were prevented by IL-6 antibody treatment. Based on these data, the purpose of this study was to determine the effects of IL-6 on the immune and neuroinflammatory responses to RSD. Our findings here support the notion that IL-6 is a critical mediator of anxiety-like behavior and social avoidance following social defeat. We provide novel evidence that IL-6 directly and robustly alters the profile of monocytes released from the bone marrow in response to social defeat stress. The molecular signature of these bone marrow–derived monocytes is associated with a primed inflammatory phenotype. Consistent with priming, monocytes from stressed mice had an exaggerated inflammatory response to immune challenge compared with control mice. This primed signature of peripheral monocytes took on an IL-6-dependent inflammatory/reactive profile following recruitment to the brain. Thus, IL-6 produced during stress is critical to the generation of primed and proinflammatory monocytes that trigger the development of anxiety-like behavior.

A key finding of this study was that stress-induced anxiety-like behavior and social avoidance were prevented in IL-6^{KO} mice. These data are consistent with other studies on social defeat stress. Administration of antibodies targeting IL-6 prevented stress-induced IL-6 production and social avoidance behavior (16). Conversely, bone marrow transplantation from socially avoidant mice induced social avoidance in naïve mice following a single exposure to stress (16). It is important to note that social avoidance behavior after RSD stress is regulated by a mechanism different from anxiety. For example, a single episode of stress does not trigger an immune response that is critical for anxiety, but it is sufficient to induce social avoidance (29). Because IL-6^{KO}-stress mice were resistant to social avoidance in our current study, it is likely that IL-6 operates on multiple levels and regulates social avoidance differentially on a neuronal circuitry level. Although the exact mechanisms underlying the effects of IL-6 on neuronal and behavioral regulation are not well understood, studies have implicated the serotonergic system in the brain. For example, increased serotonin transporter (SERT) expression is associated with dampened serotonergic signaling and mood deficits. Therefore, inhibition of SERT activity has been a target for therapy development (30). One study reported that IL-6^{KO} mice had increased SERT expression at baseline and increased serotonin uptake; however, this was associated with reduced

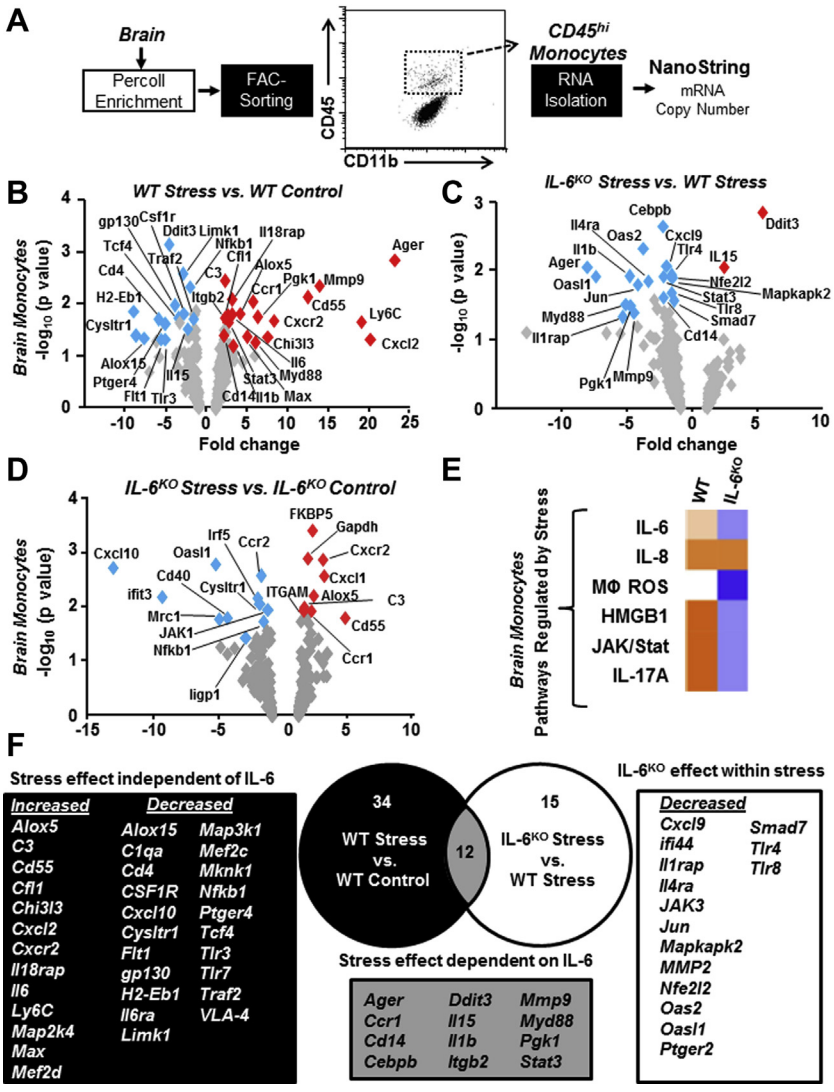

Figure 6. The primed and interleukin-1β (IL-1β) signature of monocytes recruited to the brain during stress was dependent on IL-6. Male wild-type (WT) and IL-6 knockout (IL-6^{KO}) C57BL/6 mice were subjected to six repeated cycles of social defeat (stress) or left undisturbed as control mice (n = 5). **(A)** Monocytes ($CD11b^{+}/CD45^{hi}$) in the brain were collected via fluorescence-activated cell (FAC) sorting 14 hours later. Next, RNA was isolated and messenger RNA (mRNA) copy number of 279 genes was determined using NanoString gene array. **(B–D)** Volcano plot ($-\log_{10}$ [p value] vs. fold change) of genes differentially expressed between brain monocytes from WT-stress and WT-control groups **(B)**, IL-6^{KO}-stress and WT-stress groups **(C)**, and IL-6^{KO}-stress and IL-6^{KO}-control groups **(D)**. Genes labeled in red (increased) and blue (decreased) were significantly different. **(E)** Pathways regulated by stress in the WT and IL-6^{KO} brain monocytes after stress. **(F)** Venn diagram depicts genes significantly altered between WT-stress and WT-control groups but not between IL-6^{KO}-stress and WT-stress groups (black background), genes significantly altered between WT-stress and WT-control groups and between IL-6^{KO}-stress and WT-stress groups (gray background), and genes significantly altered only between IL-6^{KO}-stress and WT- stress groups ($p < .05$ for all). HMGB1, high mobility group box 1; JAK/Stat, Janus kinase/signal transducer and activator of transcription proteins; ROS, reactive oxygen species.

propensity to anhedonic and anxiety-like behavior (31). This finding is in contrast to studies indicating higher SERT levels as a cause of anxiety-like/depressive-like behaviors (31). Others showed that peripheral IL-6 produced during stress might directly enter the brain and influence neuronal functions (20). In fact, this study also detected a monocyte population within the brain vasculature and increased blood-brain barrier permeability after social defeat stress (20). Taken together, although the specific mechanisms remain unclear, IL-6 and blood-brain barrier alterations may directly alter neurotransmitter systems and social avoidance behavior following stress.

An important finding of the current study was that the messenger RNA signature of monocytes in circulation was consistent with clinical studies on leukocytes from stressed individuals. There is an increased prevalence of circulating $CD14^{+}/CD16^{-}$ monocytes (analogous to the $CD11b^{+}/Ly6C^{hi}$ monocytes in mice) in individuals with chronic stress (12). Transcriptional analysis of blood monocytes from stressed humans revealed an increased expression of chemotaxis and inflammation-related genes and a corresponding decrease in regulatory (*CX3CR1*) genes, viral response genes, and glucocorticoid signaling genes (12,32). In posttraumatic stress disorder studies, stress-induced increases in inflammatory genes and decreases in antigen presentation molecules (HLA-DQB1 in humans) and apoptosis-related genes (*Birc2*) were detected (33). Furthermore, ex vivo LPS stimulation of peripheral monocytes from stressed individuals triggered an exaggerated production of IL-6 (12). These clinical reports on blood monocytes are consistent with our current findings on the transcriptional profile of monocytes in circulation and in the brain following stress. Here, we describe a transcriptional signature that is dependent on IL-6 produced during stress. For instance, monocytes released into circulation during stress in the WT mice had lower levels of viral response (*Tlr3, Tlr7,* and *Stat2*) and regulatory response (*Tgfb1* and *Tgfbr1*) genes and had higher levels of myeloid inflammatory response

(*Ly6C* and *Mmp9*) and glucocorticoid sequestration (*Fkbp5*) genes. Furthermore, IL-6^{KO}-stress mice had altered levels of *Stat2* and *Tgfbr* compared with WT-stress mice. These findings point to the precise transcriptional alterations caused by increased IL-6 during stress and their implications for stress-induced priming and anxiety.

Consistent with clinical studies of stress, our findings from the PBMCs in WT mice show an exaggerated immune reactivity to ex vivo LPS after stress (12). The difference in LPS response between WT and IL-6^{KO} stress mice shows that IL-6 deficiency is associated with a lack of priming of PBMCs. It is important to highlight that although we assessed the transcriptional profile of monocytes in circulation, the priming of these monocytes may also originate within the bone marrow. It is also relevant to note that IL-6^{KO}-control PBMCs produced higher levels of IL-1β compared with WT-control PBMCs following LPS treatment. This effect has been previously reported in bone marrow–derived cells where myeloid-specific deletion of IL-6 receptor α caused increased production of IL-1β (34). In that study, the absence of IL-6 receptor α signaling prevented alternative activation of myeloid cells following LPS, causing enhanced IL-1β production (34). Nevertheless, in the context of stress, we found that IL-6 deficiency during stress reduced the exaggerated inflammatory response to subsequent LPS challenge.

We also extend the clinical findings and show experimental data on the transcriptional signature of monocytes recruited to the brain during stress. Microglia activated during stress produce chemokines to recruit IL-1β-expressing monocytes that propagate IL-1 signaling into the brain via the vascular endothelium (21). Here, we show that several proinflammatory genes expressed in the brain monocytes were dependent on IL-6. For example, we show that the primed peripheral monocytes recruited to the brain during stress acquired a reactive/inflammatory phenotype characterized by elevated levels of *CD14, Ly6C, Mmp9, IL-1β,* and *Myd88.* Importantly, these transcripts were significantly reduced in the IL-6^{KO} mice exposed to stress. Notably, *Stat3,* a key transcription factor in IL-6 signaling, was increased in the brain monocytes of the WT-stress mice but not in the IL-6^{KO}-stress mice. These data indicate that the transcriptional alterations between the two groups might be mediated by IL-6-*Stat3* signaling. Although *Stat3* expression was increased in the brain monocytes after stress, this change was not evident in the blood monocytes. This may be related to the more homogeneous subpopulation (reactive/inflammatory) of monocytes that accumulate in the brain versus the more diverse monocyte population in the blood. These findings provide an in-depth view of the monocyte phenotype that is critical to the induction of anxiety-like behavior following stress.

The transcriptional signature of monocytes from a primed state in circulation to a reactive/inflammatory state in the brain is notable. This phenotypic evolution of monocytes indicates that they acquire an activated profile (IL-$1\beta^{+}$) following recruitment to the brain and interaction with the vascular microenvironment. RSD induces cell adhesion molecule (ICAM-1, VCAM, and E-Selectin) and IL-1 receptor 1 expression on the brain vasculature that provide interaction sites for monocytes recruited from the periphery (17,21,35). Notably, IL-6 deficiency did not prevent microglial activation or monocyte recruitment to the brain during stress; however, it blocked anxiety-like behavior. Thus, the presence of monocytes in the brain alone does not augment anxiety. The key event is the induction of the proinflammatory phenotype in the recruited monocytes, which we demonstrate depends on IL-6.

IL-6 also stimulates corticosterone production following specific inflammatory challenge or exogenous IL-6 administration (36,37); therefore, the effects of IL-6 deficiency on the hypothalamic-pituitary-adrenal/corticosterone response to stress is worth discussing. Although we did not test corticosterone levels in the IL-6^{KO} mice after stress, other studies have reported no change in corticosterone levels in male IL-6^{KO} mice after restraint stress (36,38). We have previously shown that corticosterone depletion via adrenalectomy or metyrapone treatment during stress reduces IL-6 production, monocyte release into circulation, and the induction of neuroendothelial ICAM-1 during stress (17). Because, in the current study, IL-6 deficiency was not associated with reduction in circulating monocytes (Figure 2F) or ICAM-1 (data not shown), the corticosterone response to stress was likely unaltered in the IL-6^{KO} mice.

A limitation of our study is that we do not prove that IL-6 is the direct cause of anxiety-like behavior following stress. Nonetheless, absence of IL-6 signaling in IL-6^{KO} mice or WT mice treated with IL-6 antibody also prevented social avoidance in a different model of RSD stress (16). Furthermore, bone marrow transplant of susceptible monocytes into naïve mice was sufficient to induce susceptibility to social avoidance in the recipient mice (16). In line with these findings, we show here that IL-6 signaling in monocytes plays a vital role in the induction of anxiety-like behavior after stress. Although the mechanism underlying IL-6-induced priming of monocytes during stress remains unknown, we demonstrate, for the first time, an IL-6-dependent transcriptional signature of monocytes that is critical to the development of anxiety following stress.

In conclusion, we show that IL-6 is vital to the priming of bone marrow–derived peripheral monocytes that are recruited to the brain during RSD and trigger anxiety-like behavior. In the absence of IL-6, monocytes in the brain showed an attenuated inflammatory profile, including reduced IL-1β expression, which was associated with overall reduced inflammatory signaling in the brain and an absence of anxiety-like behavior. These findings provide important insight into the unique monocyte profile induced by the high IL-6 levels. Understanding the transcriptional changes in immune cells during stress may allow for the development of therapeutics against inflammation-associated anxiety disorders.

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