Sympathetic Release of Spleen Monocytes Promotes Recurring Anxiety Following Repeated Social Defeat

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Conflict of interest

The authors declare no conflict of interest.

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Abstract

Background: Neuroinflammatory signaling may contribute to the pathophysiology of chronic anxiety disorders. Previous work showed that repeated social defeat (RSD) in mice promoted stress-sensitization that was characterized by the recurrence of anxiety following sub-threshold stress 24 days after RSD. Furthermore, splenectomy following RSD prevented the recurrence of anxiety in stress-sensitized (SS) mice. We hypothesize that the spleen of RSD-exposed mice became a reservoir of primed monocytes that were released following neuroendocrine activation by sub-threshold stress.

Methods: Mice were subjected to sub-threshold stress (i.e., single cycle of social defeat) 24 days after RSD, and immune and behavioral parameters were then determined.

Results: Sub-threshold 24 days after RSD re-established anxiety-like behavior that was associated with egress of Ly6C<sup>hi</sup> monocytes from the spleen. Moreover, splenectomy prior to RSD blocked monocyte trafficking to the brain and prevented anxiety-like behavior following sub-threshold stress provided 24 days later. Splenectomy, however, had no effect on monocyte accumulation or anxiety when determined 14 hours after RSD. In addition, splenocytes cultured 24 days after RSD exhibited a primed inflammatory phenotype. Next, treatment with a peripheral sympathetic inhibitor prior to sub-threshold stress blocked monocyte redistribution and prevented the re-establishment of anxiety in RSD-sensitized mice.

Conclusion: The spleen served as a unique reservoir of primed monocytes that were readily released following sympathetic activation by sub-threshold stress that promoted the re-establishment of anxiety. Collectively, these data show that the spleen is capable of storing primed monocytes that promote exaggerated behavioral responses to acute stress, even many days after a sensitizing event.
Introduction

Psychological stress contributes to the development and exacerbation of mental health disturbances, especially chronic anxiety disorders [1-4]. This is an important phenomenon because chronic anxiety disorders are the most common psychiatric illness affecting nearly 1 in 3 individuals over their life span [5, 6]. Despite this, the biological underpinnings of the relationship between psychological stress and persistent anxiety disorders are not well understood. Recent evidence indicates that bidirectional communication between the brain and immune system contributes to the etiology of many psychiatric symptoms and disorders in relation to psychological stress [7-11]. Broadly, chronic psychosocial stress is associated with a sequela of immunological changes that are often correlated with poor mental health outcomes. Many of these immunological changes are related to increased accumulation of primed monocytes that have increased potential for inflammatory signaling [12, 13] that is resistant to the anti-inflammatory effects of glucocorticoids (GCs) [14, 15]. Moreover, many of the pro-inflammatory effects of stress can be attributed to enhanced monocytopoiesis in the bone marrow that results in the selective accumulation of the Ly6C<sup>hi</sup> monocyte subset [13, 16]. Ly6C<sup>hi</sup> monocytes have a higher inflammatory capacity compared to their more mature immunoregulatory Ly6C<sup>lo</sup> counterparts [17, 18]. Additionally, there is evidence that this monocytic immune activation contribute to psychiatric illness in humans, as reviewed by Beumer et al. [19]. For example, increased perivascular brain-macrophages were observed in depressed patients who committed suicide [20]. Moreover, PTSD symptoms significantly correlated with pro-inflammatory NFκB signaling in leukocytes that was related to GC-resistance in monocytes [21, 22]. Thus, these clinical data provide key evidence that links stress, monocytes, and mood disorders.

Repeated social defeat (RSD) in mice recapitulates key immunological and behavioral deficits [23, 24] associated with psychosocial stress in humans. For example, RSD increased monocytopoiesis in the bone marrow that caused selective accumulation of Ly6C<sup>hi</sup> monocytes in circulation, spleen, and brain.
The accumulation of Ly6C<sup>hi</sup> monocytes during RSD promoted a pro-inflammatory leukocyte “transcriptional fingerprint” that was similar to that observed in human populations [13]. Similarly, RSD promotes a primed monocyte phenotype characterized by enhanced expression of toll-like receptors, co-stimulatory molecules, exaggerated inflammatory response to ex vivo innate immune challenge that is resistant to inhibition by GCs [27]. Additionally, immune activation and changes in behavior have been mechanistically linked in this model. For example, the development of prolonged anxiety-like behavior that is detectable up to 8 days after RSD [28] is dependent upon sympathetic activation of the immune system [13, 25, 27]. More specific studies revealed that monocyte accumulation in the brain mediated the relationship between immune activation and prolonged anxiety-like behavior [29]. Taken together, monocyte trafficking to the brain represent a novel axis of immune-to-brain signaling that promotes prolonged behavioral responses to stress [30, 31].

Recent evidence shows that RSD caused long-term sensitization that predisposed mice to have exaggerated immunological and behavioral responses following subsequent exposure to an acute stressor [28]. In this study, RSD-exposed mice were termed “stress-sensitized” because they exhibited exaggerated responses to an otherwise sub-threshold stressor. For instance, exposure to a single cycle of social defeat 24 days after RSD re-established monocyte trafficking and anxiety-like behavior without affecting these parameters in naïve, non-stressed controls [28]. Notably, splenectomy in stress-sensitized mice prevented the re-establishment of monocyte trafficking and anxiety-like behavior 24 days after RSD. This data was interpreted to indicate that monocyte trafficking from the spleen to the brain promoted the re-establishment of anxiety in stress-sensitized mice. However, it is currently unclear if the spleen is unique in its ability to store these releasable monocytes. In immunological studies, other immune organs were capable of storing myeloid cells, but the spleen was unique in its capacity to functionally contribute monocytes to distant inflammatory sites [32-35].
Based on these collective data, the objective of this study was to test the hypothesis that the spleen of RSD-exposed mice serves as a unique reservoir of primed monocytes that are released following sympathetic outflow in response to an acute stressor. Here, we provide several lines of novel evidence that the spleen is unique in its capacity to maintain and release a population of primed monocytes 24 days after RSD. Moreover, sub-threshold stress in stress-sensitized (SS) mice caused this pool of primed monocytes to traffic to the brain and promote the recurrence of anxiety-like behavior. Furthermore, inhibition of the peripheral sympathetic nervous system during sub-threshold stress blocked spleen-to-brain monocyte trafficking and prevented the recurrence of anxiety in stress-sensitized mice. These novel studies reveal that the spleen is capable of maintaining long term neuroimmune sensitization that can regulate behavioral responses many days after the initial sensitizing event.

Materials and Methods

Mice. Male C57BL/6 (6–8 weeks old) and CD-1 (retired breeders) mice were purchased from Charles River Laboratories (Wilmington, MA) and allowed to acclimate to their surroundings for 7–10 d before initiation of any experimental procedures. C57BL/6 mice were housed in cohorts of three per cage. All procedures were in accordance with the NIH 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 (RSD). Mice were subjected to RSD as previously reported [29] and as described in Supplementary Materials. In brief, an aggressive intruder male CD-1 mouse was introduced into cages of established male cohorts (three per cage) of C57BL/6 mice for 6 consecutive nights. During each cycle, submissive behaviors were observed to ensure that the resident mice showed subordinate behavior. At the end of the 2 h period, the intruder was removed and the residents were left undisturbed until the following day when the paradigm was repeated. As previously described [28], to study the sensitizing effects of
RSD, mice were either exposed to control (naïve) or RSD conditions (stress-sensitized). Then, 24 days later naïve and stress-sensitized (SS) mice were subjected to an additional cycle of social defeat. All behavior and biological measures were obtained 14 h after the final cycle.

Guanethidine Treatment. Twenty four hours prior to acute social defeat, mice were injected subcutaneously with either vehicle or 50 mg/kg guanethidine (Santa Cruz Biotechnology, Dallas, TX). Injection regimen was based on a previous report [36].

Anxiety-like behavior. Anxiety-like behavior was determined using open-field activity as previously reported [29] and as described in Supplementary Materials.

Isolation of cells from bone marrow, spleen, blood, and brain. Tissues were collected immediately following CO₂ asphyxiation. Cells from BM, spleen, and blood were isolated as previously described [26, 27]. CD11b⁺ brain cells were enriched by Percoll density gradient as previously reported [29]. See Supplementary Materials for details.

Ex vivo culture with LPS and corticosterone. As previously reported [27, 37], BM and spleen cells were treated with 1 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0127:B8, Sigma-Aldrich, St. Louis, Missouri) and various concentrations of corticosterone (Sigma-Aldrich). Cells were incubated 18 hours for supernatant cytokine measurements or 48 hours for assessment of cell viability. Cell viability was determined by CellTiter 96 Non-radioactive Proliferation Assay (Promega; Madison, WI), and supernatant IL-6 was determined by ELISA (BD Biosciences; San Diego, CA). See Supplementary Materials for details.

Statistical analysis. To determine significant main effects and interactions between main factors, data were analyzed using two-way ANOVA using the General Linear Model procedures of SAS (Cary, NC). ANOVA results are presented in figure legends. When there was a main effect of experimental treatment
or a treatment interaction effect, differences between means were evaluated by an $F$-protected t-test using the Least-Significant Difference procedure of SAS. All data are expressed as treatment means ± SEM.

Results

Recurrence of anxiety-like behavior in stress-sensitized mice was associated with Ly6C$^{hi}$ monocyte egress from the spleen

Previous studies showed that removal of the spleen after RSD prevented both monocyte trafficking to the brain and the recurrence of anxiety-like behavior in stress-sensitized (SS) mice [28]. To further examine the possible release of monocytes from the spleen in response to acute stress, the following experimental design was used. Fig.1A illustrates that mice were stress-sensitized by 6 cycles of social defeat (SS) or left undisturbed (Naïve). Twenty four days later, mice were exposed to acute social defeat (acute stress) or left alone as controls. Congruent with our previous findings [28], acute social defeat promoted the recurrence of anxiety-like behavior that was associated with increased monocyte trafficking to the brain. For instance, SS mice exposed to acute stress took longer to enter the center (Fig.1B, $p<0.05$) and spent less time in the center of the open field (Fig.1C, $p<0.05$). Additionally, acute stress increased the accumulation of macrophages in the brain (Fig.1D, $p<0.05$) and increased mRNA expression of IL-1β, TNF-α, and CD14 in the brain of SS mice (Fig.1E, all $p<0.05$). Next, the peripheral origin of increased brain macrophages was explored. This revealed that acute stress increased the number of circulating Ly6C$^{hi}$ monocytes in SS mice (Fig.1F, $p<0.05$) but not in naïve mice. Additionally, the increase in circulating monocytes was associated with increased release of monocytes from the spleen (Fig 1G, $p<0.05$) and bone barrow of SS mice (Fig.1H, $p<0.05$). Neither stress-sensitization nor acute stress altered spleen weight (Fig.1I). These results demonstrated that the spleen is indeed a primary source of monocyte accumulation in SS mice following exposure to acute stress.
Splenectomy prior to RSD prevented recurrence of monocyte trafficking and anxiety-like behavior following acute stress in sensitized mice

Previous studies show that removal of the spleen after RSD prevents monocyte accumulation in the brain following acute stress 24 days later [28]. It is possible, however, that other immune compartments may compensate for the spleen following splenectomy and function as alternative myeloid reservoirs [35]. Thus, the next objective was to determine if other immune reservoirs can compensate for the spleen during stress. To do this, mice were splenectomized 14 days prior to RSD and then exposed to acute social defeat 24 days later (Fig.2A). This showed that indeed splenectomy prior to RSD prevented the recurrence of monocyte trafficking and anxiety-like behavior in SS mice. For instance, acute stress in sham-treated SS increased Ly6C<sup>hi</sup> monocytes in circulation (Fig.2B-C, \( p<0.05 \)), increased brain-macrophages (Fig.2D-E, \( p<0.05 \)), and increased IL-1β, TNF-α, and CD14 mRNA expression in the brain (Fig.2F, all \( p<0.1 \)), and all of these effects were prevented by splenectomy (Fig.2B-F). Moreover, prevention of monocyte trafficking to the brain corresponded with prevention of the recurrence of anxiety-like behavior. For instance, Sham-SS mice exhibited increased time to enter the center (Fig.2H, \( p=0.1 \)) and reduced time spent in the center (Fig.2I, \( p<0.05 \)) of the open field, while these anxiety-like behaviors were not detected in splenectomized SS mice (Fig.2H&I). These data are interpreted to indicate that other immune compartments were unable to compensate for the spleen and act as functional reservoirs of releasable monocytes following RSD.

Splenectomy did not influence monocyte trafficking or anxiety-like behavior 14 hours after RSD

Our data indicate that monocyte release and anxiety-like behavior following acute stress in SS mice was dependent on the spleen (Fig.2). It is possible that the spleen was also necessary for some of the primary immune and behavioral responses to the initial exposure to RSD. To address this, mice were splenectomized prior to RSD, and behavioral and biological measures were determined 14 hours after the final cycle (Fig.3A). This showed that the primary immune and behavioral responses to RSD were...
unaltered by splenectomy. Consistent with substantial accumulation of primed myeloid cells in the spleen [26], RSD increased spleen weight in sham mice (Fig. 3B; $p<0.05$). Independent of splenectomy, RSD enhanced myelopoiesis (monocytes and granulocytes) and decreased lymphopoiesis and erythropoiesis in the bone marrow (Fig.3C-D; all $p<0.05$). Moreover, splenectomy did not prevent increased Ly6C$^{hi}$ monocytes in circulation following RSD (Fig.3E, $p<0.05$), did not prevent the accumulation of macrophages in the brain (Fig.3F-G, $p<0.05$), and did not prevented increased brain cytokine mRNA expression of IL-1$\beta$, IL-6, TNF-α, CD14, and CCL2 (data not shown) that all occurred independently of splenectomy. In addition, splenectomy did not prevent the development of anxiety-like behavior 14 hours after RSD. For instance, RSD increased time to enter the center (Fig.3H, $p<0.05$) and decreased time spent in the center (Fig. 3I, $p<0.05$) of the open field independent of splenectomy. Taken together the spleen was not required for the primary immune and behavioral response to RSD observed 14 hours after the last cycle.

RSD increased the accumulation of primed myeloid cell in the spleen that exhibited exaggerated inflammatory response to ex vivo mitogen challenge

Data presented here demonstrate that the spleen is necessary for the maintenance of a releasable pool of monocytes following RSD. Previous reports indicated that RSD increased release and trafficking of BM-derived monocyte-lineage cells that are both primed and GC-insensitive [26, 37, 38]. For instance, previous studies showed that RSD caused myeloid cells to produce exaggerated levels of IL-6 in response to ex vivo LPS stimulation, and this was associated with resistance to the pro-apoptotic effects of GC stimulation [26, 27]. Additionally, previous results indicated that these primed monocytes seed the spleen and retain a GC-insensitive phenotype for at least 8 days after RSD [39]. Nonetheless their presence and phenotype at 24 days after RSD is unknown. To address this, spleen and BM cells were collected from SS mice and reactivity to LPS and GC sensitivity were assessed ex vivo 24 days after RSD (Fig.4A). IL-6 production following ex vivo LPS stimulation of BM was not different between groups (Fig.4B).
However, splenocytes from SS mice produced more IL-6 following LPS stimulation compared to cells from naïve mice (Fig.4C, P<0.05). Additionally, there was increased cell viability in response to LPS stimulation in splenocytes from SS mice compared to those from naïve mice (Fig. 4D p<0.05). This exaggerated splenocyte response to LPS stimulation was associated with enhanced baseline mRNA expression of CD14 (Fig. 4E, p<0.05) but not TLR4 (data not shown). Next, to determine if this primed phenotype was associated with GC-insensitivity in SS mice, the effect of increasing corticosterone concentrations on LPS-induced IL-6 production and cell viability was determined. Fig.4G&F show that increasing corticosterone concentrations reduced cell viability (Fig.4F, p<0.05) and IL-6 production (Fig.4G, p<0.05) independent of stress-sensitization. Thus, primed but not GC-insensitive monocytes were maintained in the spleen for at 24 days after RSD.

**Sympathetic inhibition prevented monocyte trafficking and the recurrence of anxiety-like behavior in SS mice**

Data shown here demonstrate that the spleen is uniquely responsible for the increased availability of primed and releasable monocytes 24 days after RSD. Despite this, the physiological signaling pathway that initiates release of monocytes from the spleen in response to acute stress was unknown. Previous studies demonstrate a role for the SNS in the release of splenic myeloid cells [40]. Therefore, the effect of guanethidine, a peripheral sympathetic inhibitor, on monocyte release was determined (Fig.5A). Guanethidine displaces norepinephrine from its vesicles, thus preventing its release in a dose-dependent manner [41]. This study showed that similar to splenectomy, sympathetic inhibition with guanethidine prevented monocyte trafficking and anxiety-like behavior in SS mice. For instance, acute stress in vehicle-treated SS mice increased Ly6C<sup>hi</sup> monocytes in circulation (Fig.5B&C, p<0.05) and increased CD45<sup>hi</sup> brain-macrophages (Fig.5D&E, p<0.05). This redistribution of monocytes was not detected in guanethidine-treated SS mice (Fig.5B-E). Similarly, acute stress in vehicle-treated SS mice increased brain mRNA expression of IL-1β and TNF-α (Fig.3F, both p<0.05), and this was also prevented by
guanethidine treatment. Moreover, blockade of monocyte trafficking to the brain with guanethidine
corresponded with prevention of anxiety-like behavior in SS mice. For instance, acute stress in vehicle-
treated SS mice increased time to enter the center (Fig.5H, p<0.05) and reduced time spent in the center of
the open field (Fig.5I), and these behaviors were not observed in guanethidine treated SS mice
(Fig.5H&I). Taken together, sympathetic inhibition prevented spleen-to-brain monocyte trafficking, and
this corresponded with attenuated anxiety-like behavior and reduced neuroinflammatory signaling
following acute stress exposure.

Discussion

The results presented here demonstrate a novel and critical role for the spleen in the maintenance
of stress-sensitization that persisted for many days after the initial sensitizing, stress event. First it is
shown that the recurrence of anxiety-like behavior is associated with increased monocyte trafficking from
the spleen and increased macrophage accumulation in the brain. Next, novel data shown here indicate that
the spleen was indispensable for the maintenance of primed and releasable monocytes 24 days after RSD.
For example, splenectomy prior to stress-sensitization blocked monocyte re-distribution and prevented the
recurrence of anxiety in stress-sensitized mice. Notably, no other organ acted as a compensatory reservoir.
Additionally, splenectomy prior to RSD did not attenuate the primary immune and behavioral response to
RSD observed at 14 hours after the final cycle. Thus, the spleen was necessary for the maintenance of
releasable monocytes 24 days after RSD, but was not necessary for the initial production and trafficking
of primed monocytes or anxiety 14 hours after the initial exposure to RSD. The next experiment showed
that splenic monocytes retained a primed but not GC-insensitive phenotype in stress-sensitized mice. This
was interpreted to indicate that RSD primed and mobilized monocyte-lineage cells that persisted in the
spleen for 24 days following cessation of the stressor. Further work addressed physiological signals that
contributed to the release of monocytes from the spleen. These studies showed that pretreatment with the
SNS-inhibitor, guanethidine, prevented monocyte trafficking and anxiety in stress-sensitized mice. Thus,
sympathetic initiation of spleen-to-brain monocyte trafficking promoted the recurrence of anxiety-like behavior in sensitized mice.

An important finding in this study was that stress-sensitization following RSD was associated with an altered myeloid composition of the spleen. First, there was a tendency for increased Ly6C^{hi} monocytes in the spleen that persisted 24 days after exposure to RSD. Second, accumulation of monocytes in circulation and brain following acute stress in stress-sensitized mice was associated with a robust reduction in the number of Ly6C^{hi} monocytes in the spleen. This is consistent with egress of Ly6C^{hi} monocytes from the spleen that accumulated in circulation and brain. This re-distribution of splenic monocytes characterized here resembles studies of myocardial infarction that revealed that monocyte re-distribution from the spleen contributed to myocardial pathogenesis [32]. Notably, acute stress in stress-sensitized mice also reduced the number of Ly6C^{hi} monocytes in the BM. Nonetheless, our previous work [28] and data presented here show that cells from the spleen but not the BM are critical for increased trafficking of primed monocytes in stress-sensitized mice.

The splenectomy studies presented here provide evidence that the spleen is not required for primary immune and behavioral responses to RSD, but rather, the spleen is necessary for the maintenance of releasable monocytes 24 days after RSD. This is an important distinction, because it implicates the BM and not the spleen in the initial production and accumulation of monocytes immediately following RSD. These results are consistent with other studies of RSD and chronic unpredictable stress that demonstrated increased production of myeloid cells in the BM [16, 26]. Data from RSD indicated that the monocytes that accumulate with stress are primed to be more inflammatory in response to challenges (e.g., LPS) and less sensitive to the anti-inflammatory effects of GCs [27]. Thus, we hypothesize that RSD mobilizes primed monocytes that seed the spleen and contribute to the maintenance of releasable monocytes with the ability to traffic in the brain and promote anxiety in stress-sensitized mice.
Related to the above points, data here support the hypothesis that splenic monocytes from stress-sensitized mice are inherently more reactive to neuroendocrine or immune stimulation. For instance, cells that persist in the spleen 24 days after RSD appear to have a more primed profile with increased IL-6 secretion following ex vivo LPS stimulation. In contrast, BM cells from stress-sensitized mice were not more sensitive to LPS stimulation. It is important to mention that these experiments were completed with whole splenocytes, but we attribute these affects to monocytes. This is supported by previous studies showing that monocytes/macrophages were the primary cells that responded to LPS stimulation in ex vivo splenocyte cultures [42]. Although stress-sensitized mice retained a primed monocyte phenotype, they did not retain the GC-insensitive phenotype that is observed for up to eight days after RSD [27, 39]. We interpret these data to indicate that the spleen maintains a population of primed monocytes following stress-sensitization and that these cells can traffic to the brain and promote the recurrence of anxiety following acute stress many days later. Despite the evidence provided here, it is possible that enhanced splenic monocyte trafficking observed in stress-sensitized mice is mediated by neuroendocrine sensitization and was unrelated to immunomodulation. For example, fear conditioning in stress-sensitized mice might contribute to exaggerated neuroendocrine response to the acute stressor, resulting in sufficient stimulation to cause the release of splenic monocytes that traffic to the brain and promote anxiety. Nonetheless, priming of splenic monocytes was observed independent of neuronal mediation. For instance, splenic myeloid cells demonstrated increased CD14 mRNA expression and enhanced IL-6 production following ex vivo LPS stimulation. Thus persistent splenic priming was observed independent of neuroendocrine sensitization.

Another important finding was that the release of primed monocytes from the spleen of stress-sensitized mice after acute social defeat was dependent on the SNS. The SNS can interact with the spleen either through circulating epinephrine or norepinephrine released from the adrenal medulla or through direct sympathetic innervation [43]. Guanethidine is a peripheral SNS inhibitor that displaces
norepinephrine from its vesicles and does not affect the CNS [41]. Experiments completed here show that
monocyte release from the spleen was dependent upon SNS activation. For example, guanethidine
blocked accumulation of Ly6C<sup>hi</sup> monocytes in circulation and blocked macrophage trafficking in the
brain. Notably, this blockade corresponded with prevention of anxiety-like behavior in stress-sensitized
mice. This point is of particular interest because it reveals a clinically relevant pharmacological strategy to
attenuate maladaptive behaviors related to peripheral immunological sensitization. Although
underappreciated, it has been reported that β-adrenergic antagonists (i.e., beta-blockers) have chronic
anxiolytic effects in certain clinical populations [44] that may be related to interactions with the immune
system. Thus, studies here provide a biological mechanism that supports the use of sympathetic inhibitors
to abrogate recurring anxiety promoted by monocyte redistribution.

Overall, the current studies provide evidence that the spleen contributes to long term neuroimmune
sensitization capable of regulating behavioral responses many days after a sensitizing stressful event. For
example, the spleen acted as unique reservoir for primed monocytes following exposure to RSD that were
readily releasable following neuroendocrine activation by acute stress 24 days later. Neuroendocrine
activation by acute stress caused primed monocytes to traffic to the brain and promote the recurrence of
anxiety in sensitized mice. This phenomenon may be relevant because persistent or recurring behavioral
complications observed in several psychiatric populations are associated with immune activation [45].
Thus, recurring behavioral complications associated with psychological stress may be related to splenic
monocyte re-distribution. Collectively, these findings reveal novel neuroimmune mechanisms that may be
implicated in recurring anxiety disorders.
Figure Legends:

Figure 1. Acute stress in stress-sensitized (SS) mice caused re-establishment of anxiety-like behavior that was associated with release monocytes from the spleen A) Male C57BL/6 mice were stress-sensitized (SS) by 6 repeated cycles of social defeat or left undisturbed as controls (Naïve). Mice were subjected to acute social defeat 24 days later and anxiety-like behavior and biochemical analyses were completed 14 h later. Stress-Sensitized mice exposed to acute social defeat exhibited anxiety-like behavior in the open field with B) increased time to enter the center (interaction, $F_{1,42}=4.52$, $p<0.05$) and C) reduced time spent in the center (tendency for interaction, $F_{1,44}=2.98$, $p<0.10$). D) Acute social defeat in SS mice increased percentage of macrophages associated with the brain (interaction, $F_{1,21}=8.22$, $p<0.05$) and E) increased Ly6C$^{hi}$ monocytes in circulation (main effect of SS, $F_{1,19}=4.47$, $p\leq0.05$; tendency for interaction, $F_{1,19}=2.67$, $p\leq0.1$). F) Several inflammatory mediators were determined in a coronal brain section and acute social defeat increased mRNA expression of IL-1β in SS mice ($F_{1,36}=10.55$, $p<0.01$; interaction, $F_{1,36}=3.66$, $p\leq0.05$), CCL2 ($F_{1,36}=5.42$, $p<0.05$), TNF (interaction, $F_{1,39}=4.23$, $p<0.05$), and CD14 (interaction, $F_{1,39}=4.46$, $p<0.05$). The relative number of Ly6C$^{hi}$ monocytes was determined in the G) spleen and H) bone marrow. Acute stress reduced the number of monocytes in both the spleen (interaction, $F_{1,18}=8.35$, $p<0.01$) and bone marrow (interaction, $F_{1,18}=9.82$, $p<0.01$) of SS mice. I) Spleen weight was determined and shown as a percentage of body mass. Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON ($p<0.05$) according to F-protected post hoc analysis.

Figure 2. Splenectomy prior to stress-sensitization prevented re-establishment of monocyte trafficking and anxiety-like behavior following subsequent exposure to acute stress. A) Male C57BL/6 mice were subjected to sham or splenectomy (SPLX) surgery and were allowed to recover for 14 days. Mice were then stress-sensitized (SS) by 6 repeated cycles of social defeat or left undisturbed as controls (Naïve). Twenty four days later, mice were subjected to acute social defeat, anxiety-like behavior...
and biochemical analyses were completed 14h later. **B**) Representative flow Bi-variate dot plots of CD115 and Ly6C labeling of blood cells. **C**) The percentage of Ly6C^{hi} monocytes was determined in blood. Monocytes in circulation were increased by acute stress in SS mice ($F_{1,25}=11.9, p<0.05$) and this effect was blocked by splenectomy (tendency for interaction, $F_{1,25}=3.3, p<0.1$). **D**) Representative flow Bi-variate dot plots of CD11b and CD45 labeling on enriched brain macrophages (MΦ) and microglia (MGL). **E**) The percentage of brain macrophages was determined and they were increased by acute stress ($F_{1,25}=2.9, p<0.1$) and this effect was blocked by splenectomy (tendency for interaction, $F_{1,25}=3.52, p<0.1$). **F**) Several inflammatory mediators were determined in a coronal brain section and acute social defeat increased mRNA expression of IL-1b ($F_{1,18}=2.4, p<0.1$), TNFa ($F_{1,18}=2.83, p<0.1$) and CD14 ($F_{1,18}=7.92, p<0.05$) in Sham mice but not SPLX mice. **G**) Spleen weight is shown. Stress-sensitized Sham mice exhibited anxiety-like behavior in the open field with increased time to enter the center (**H**; tendency for main effect of sensitization, $F_{1,25}=3.2, p<0.1$) and reduced time spent in the center (**I**; interaction effect, $F_{1,25}=6.5, p<0.05$). Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON ($p<0.05$) and means with (#) tended to be different from CON ($p<0.1$), according to F-protected post hoc analysis.

**Figure 3.** Splenectomy did not influence myelopoiesis, monocyte redistribution, or the establishment of anxiety-like behavior following initial exposure to RSD. **A**) Male C57BL/6 mice were subjected to sham or splenectomy (SPLX) surgery and were allowed to recover for 14 days. Mice were then exposed to repeated social defeat (RSD) or left undisturbed as controls (CON), and 14 hrs after the final cycle, anxiety-like behavior was assessed in the open field. Subsequently, brain, blood, and bone marrow were collected for analysis. **B**) RSD increased spleen weight in sham mice ($p<0.05$). Spleen weights were not detectable (n.d.) in splenectomized mice. **C**) Representative flow Bi-variate dot plots of CD31 and Ly6C labeling on bone marrow cells is shown. **D**) Independent of splenectomy, RSD decreased erythrocytes ($F_{1,17}=124.5, p<0.0001$) and lymphocytes ($F_{1,17}=129.2, p<0.01$) and increased monocytes ($F_{1,17}=120.0,$
p<0.0001) and granulocytes ($F_{1,17}$=144.9, $p<0.0001$) in bone marrow. E) RSD increased percent Ly6C$^{hi}$ monocytes in circulation independent of splenectomy ($F_{1,18}$=12.0, $p<0.01$). F) Representative flow Bi-variate dot plots of CD11b and CD45 labeling on enriched brain macrophages (MΦ) and microglia (MGL). G) RSD increased percent brain macrophages ($F_{1,18}$=10.4, $p<0.01$). RSD increased anxiety-like behavior in the open field with increased time to enter the center (H; $F_{1,23}$=8.2, $p<0.01$) and decreased time spent in the center (I; $F_{1,23}$=10.2 $p<0.01$). Abbreviations: Mo, Monocytes; Gr, Granulocytes; Ly, Lymphocytes; Er, Erythrocytes. Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON ($p<0.05$) and means with (#) tended to be different from CON ($p<0.1$), according to $F$-protected post hoc analysis.

Figure 4. Stress-sensitization resulted in the accumulation of primed splenocytes with an enhanced response to ex vivo mitogen challenge  A) Male C57BL/6 mice were stress-sensitized (SS) by 6 repeated cycles of social defeat or left undisturbed as controls (Naïve). Twenty four days later, spleen and bone marrow (BM) cells were cultures ex vivo in the presence of lipopolysaccharide (LPS) and corticosterone (cort). IL-6 protein was determined in the cell supernatants collected 18 h after LPS in BM and Splenocytes. B) IL-6 secretion was similar between groups in bone marrow cells. C) The LPS induced IL-6 secretion was higher in splenocytes cultured from SS mice compared to naïve mice ($p<0.01$). D) Cell viability of LPS-stimulated splenocytes was increased in SS mice ($p<0.05$). E) mRNA expression of CD14 in SS splenocytes was higher than naïve ($p<0.01$). Next, ex vivo cultures from spleen were stimulated with LPS for 48 h in the presence of increasing concentrations of corticosterone and cell viability and IL-6 concentrations were determined. F) There was a main effect of corticosterone on viability ($F_{5,36}$=6.84, $p<0.0001$) and G) on IL-6 production $F_{5,36}$=4.42, $p<0.005$) that was independent of stress. F&G) There was also a main effect of stress on viability ($F_{1,36}$=9.63, $p<0.005$) and IL-6 production ($F_{1,36}$=4.69, $p<0.05$). Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON ($p<0.05$) according to $F$-protected post hoc analysis.
Figure 5. Guanethidine blocked primed monocyte trafficking from the spleen to the brain and prevented the re-establishment of anxiety in stress sensitized mice. A) Male C57BL/6 mice were stress-sensitized (SS) by 6 repeated cycles of social defeat or left undisturbed as controls (Naïve). Mice were pretreated with guanethidine (Guan) or Vehicle (Veh) prior to acute social defeat. Fourteen hours after acute social defeat, anxiety-like behavior and biochemical analyses were completed. B) Representative flow Bi-variate dot plots of CD115 and Ly6C labeling on blood cells. C) The percentage of Ly6C\textsuperscript{hi} monocytes was determined in blood. Acute stress increased percent Ly6C\textsuperscript{hi} monocytes in SS-Veh mice but not naïve mice (interaction effect, $F_{1,46}=4.35$, $p<0.05$). D) Representative flow Bi-variate dot plots of CD11b and CD45 labeling on enriched brain macrophages (MΦ) and microglia (MGL). E) The percentage of brain macrophages was determined and they were increased by acute stress ($F_{1,46}=14.66$, $p<0.0005$) and this effect was blocked by guanethidine ($F_{1,46}=7.72$, $p<0.01$). F) Several inflammatory mediators were determined in a coronal brain section and acute social defeat increased mRNA expression of IL-1b ($F_{1,22}=6.46$, $p<0.05$) and TNFa ($F_{1,22}=3.18$, $p<0.1$) in SS-Veh mice but not SS-Guan mice. G) Spleen weight is shown. SS vehicle treated mice exhibited anxiety-like behavior in the open field with increased time to enter the center ($H$; $F_{1,46}=2.42$, $p\leq0.1$; main effect of splenectomy; $F_{1,46}=5.66$, $p<0.05$) and reduced time spent in the center ($I$; tendency for interaction effect, $F_{1,46}=2.42$, $p\leq0.1$). Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON ($p<0.05$) and means with (#) tended to be different from CON ($p<0.1$), according to $F$-protected post hoc analysis.
References


