Archival Report

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

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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 subthreshold stress 24 days after RSD. Furthermore, splenectomy following RSD prevented the recurrence of anxiety in stress-sensitized mice. We hypothesize that the spleen of RSD-exposed mice became a reservoir of primed monocytes that were released following neuroendocrine activation by subthreshold stress.

METHODS: Mice were subjected to subthreshold stress (i.e., single cycle of social defeat) 24 days after RSD, and immune and behavioral measures were taken.

RESULTS: Subthreshold stress 24 days after RSD re-established anxiety-like behavior that was associated with egress of Ly6C^{hi} monocytes from the spleen. Moreover, splenectomy before RSD blocked monocyte trafficking to the brain and prevented anxiety-like behavior following subthreshold stress. 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. Peripheral sympathetic inhibition before subthreshold stress blocked monocyte trafficking from the spleen to the brain and prevented the re-establishment of anxiety in RSD-sensitized mice. Last, β-adrenergic antagonism also prevented splenic monocyte egress after acute stress.

CONCLUSIONS: The spleen served as a unique reservoir of primed monocytes that were readily released following sympathetic activation by subthreshold stress that promoted the re-establishment of anxiety. Collectively, the long-term storage of primed monocytes in the spleen may have a profound influence on recurring anxiety disorders.

Keywords: Anxiety, Macrophages, Microglia, Neuroinflammation, PTSD, Stress

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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). 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 sequelae 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) and are 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^{hi} monocyte subset (13,16). Ly6C^{hi} monocytes have a higher inflammatory capacity compared with their more mature immunoregulatory Ly6C^{lo} counterparts (17,18). Additionally, there is evidence that this monocytic immune activation contributes 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, posttraumatic stress disorder symptoms significantly correlated with pro-inflammatory NFκB signaling in leukocytes and with 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^{hi} monocytes in circulation, spleen, and brain (25,26). The accumulation of Ly6C^{hi} monocytes during RSD promoted a pro-inflammatory leukocyte transcriptional fingerprint that was similar to that observed in human

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populations (13). Similarly, RSD promotes a primed monocyte phenotype characterized by exaggerated inflammatory response to ex vivo innate immune challenge that is resistant to inhibition by GCs (27). Additionally, 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). Further studies revealed that the development of prolonged anxiety-like behavior was specifically dependent on monocyte accumulation in the brain following RSD (29). Taken together, monocyte trafficking to the brain represents 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 caused 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 subthreshold 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 control mice (28). Notably, splenectomy in stress-sensitized mice prevented the re-establishment of monocyte trafficking and anxiety-like behavior 24 days after RSD. These data were 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 evidence that the spleen is unique in its capacity to maintain and release a population of primed monocytes 24 days after RSD. Moreover, subthreshold stress in 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 subthreshold 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.

METHODS AND MATERIALS

Mice

Male C57BL/6 (6–8 weeks old) and CD-1 (retired breeders) mice were purchased from Charles River Laboratories (Wilmington, Massachusetts). C57BL/6 mice were housed in cohorts of three per cage. All procedures were in accordance with the National Institutes of Health Guidelines and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Repeated Social Defeat

Mice were subjected to RSD as previously reported (29) and as described in Supplement 1. 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. As previously described (28), to study the sensitizing effects of RSD, mice were either exposed to control (naïve) or RSD conditions (stress-sensitized [SS]). Then, 24 days later naïve and SS mice were subjected to an additional cycle of social defeat. All behavior and biological measures were obtained 14 hours after the final cycle. This time point was selected because both hypothalamus-pituitary-adrenal axis and sympathetic nervous system (SNS) activation following social defeat return to baseline within 14 hours (27).

Guanethidine Treatment

Twenty-four hours before acute social defeat, mice were injected subcutaneously with either vehicle or 50 mg/kg guanethidine (Santa Cruz Biotechnology, Dallas, Texas). 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 Supplement 1.

Isolation of Cells from Bone Marrow, Spleen, Blood, and Brain

Tissues were collected immediately following carbon dioxide asphyxiation. Cells from bone marrow (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 the Supplement for details.

Statistical Analysis

To determine significant main effects and interactions between main factors, data were analyzed using two-way analysis of variance using the general linear model procedures of SAS (SAS Institute Inc., Cary, NC). Analysis of variance 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

Our previous study 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