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Repeated social defeat stress induces neutrophil mobilization in mice: maintenance after cessation of stress and strain-dependent difference

Short running title: Stress-induced neutrophil mobilization and its maintenance

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What is already known

- Stress-induced mobilization of leukocytes and their cytokine secretion are crucial for depression- and anxiety-like behaviors.

What this study adds

- Social defeat stress-induced increase in circulating neutrophils was maintained after the stress in mice.
- This increase in circulating neutrophils was larger in a mouse strain with higher stress susceptibility.

What is the clinical significance

- Stress-induced increase in circulating neutrophils and its maintenance might be relevant to stress-related mental illnesses.

Abstract

Background and Purpose

Inflammation has been associated with stress-related mental disturbances. Rodent studies have reported that blood-borne cytokines are crucial for stress-induced changes in emotional behaviors. However, roles and regulations of leukocytes in chronic stress remain unclear.

Experimental Approach

Adult male C57BL/6N mice were subjected to repeated social defeat stress (R-SDS) with two protocols which differed in stress durations, stress cycles and housing conditions, followed by the social interaction test. The numbers of leukocyte subsets in the bone marrow, spleen, and blood were determined by flow cytometry shortly or at several days after R-SDS. Two strains of mice with different stress susceptibility, C57BL/6N and BALB/c mice, were compared for these leukocyte changes.

Key Results

R-SDS with both protocols similarly induced social avoidance in C57BL/6N mice. In the bone marrow, neutrophils and monocytes were increased, and T cells, B cells, NK cells and dendritic cells were decreased with both protocols. In the blood, neutrophils and monocytes were increased with both protocols, whereas T cells, B cells, NK cells and dendritic cells were decreased with one of these. Neutrophils and monocytes were also increased in the spleen. Not only the changes in the bone marrow but also the increase in circulating neutrophils was maintained for 6 days after R-SDS. BALB/c mice showed larger social avoidance and increase in circulating neutrophils than

C57BL/6N mice.

Conclusion and Implications

These findings suggest that chronic stress induces neutrophil mobilization and its maintenance, relating to genetic variability of stress susceptibility that underlies the pathology of mental illnesses.

Abbreviations: ANOVA, analysis of variance; BMEF, bone marrow extracellular fluid; CCL2, C-C motif chemokine 2; CCR2, C-C chemokine receptor type 2; CXCL12, C-X-C motif chemokine 12; CXCR4, C-X-C chemokine receptor type 4; G-CSF, Granulocyte Colony Stimulating Factor; MFI, mean fluorescent intensity; R-SDS, repeated social defeat stress; WBC, white blood cell.

Introduction

Stress is a strain of mental and physical functions caused by adverse and demanding conditions. Although stress mediates adaptive biological responses to promote survival and well-being, excessive or prolonged stress may increase the risks of mental and physical illnesses (Yaribeygi et al., 2017; McEwen et al., 2017). The levels of cytokines and chemokines in the blood and cerebrospinal fluid are changed in patients of mental illnesses including depression (Syed et al., 2018; Wang AK et al., 2018; Miller et al., 2016; Enache et al., 2019). Studies with brain PET imaging have suggested neuroinflammation in prefrontal cortices of depressive patients (Setiawan et al., 2015). Corroborating these clinical findings, chronic stress in rodents, such as chronic mild stress and repeated social defeat stress (R-SDS), induces microglial activation in distinct brain areas such as the medial prefrontal cortex and hippocampus, and promotes concomitant neuronal and behavioral changes (Tanaka et al., 2012; Nie et al., 2018; Kreisel et al., 2014; Wohleb et al., 2011; Franklin et al., 2018; Wohleb et al., 2018). In addition, rodent studies using bone marrow transplantation and peripheral administration of neutralizing antibodies have shown that R-SDS-induced mobilization of leukocytes and their secretion of cytokines, IL-1β and IL-6, are crucial for depression- and anxiety-like behaviors (Ménard et al., 2017; Weber et al., 2017; McKim

et al., 2018; Hodes et al., 2014). To promote the entry of blood-borne cytokines to the brain parenchyma, R-SDS impairs the integrity of the blood-brain barrier (Menard et al., 2017).

Multiple R-SDS protocols with distinct behavioral readouts have been reported. In one protocol, R-SDS is applied by introducing a mouse to be defeated to the home cage of a resident aggressor mouse for 10 min daily for 10 consecutive days (hereinafter called Protocol 1). In another protocol, R-SDS is applied by introducing an aggressor mouse to the home cage of established male cohorts (three per cage) for 2 h daily for 6 consecutive days (hereinafter called Protocol 2). It has been reported that R-SDS with Protocol 2 induces neutrophils and monocytes and decreases lymphocytes in the bone marrow and blood of C57BL/6N mice (e.g., Wohleb et al., 2013; McKim et al., 2018), although whether these leukocyte changes occur with R-SDS with Protocol 1 has not been examined.

Since chronic stress including R-SDS increases neutrophils and monocytes, they may act as the cellular sources of cytokines responsible for concomitant behavioral changes. However, stress-induced mobilization of leukocyte subsets and its relevance to stress susceptibility have not been analyzed in depth. In this study, we found that R-SDS with these two protocols increases circulating neutrophils, and that this increase was maintained after R-SDS. We also analyzed its relationship to depressive-like behavior as measured by social avoidance, which has been frequently used to measure individual variability of stress susceptibility (Nestler and Hyman, 2010).

Methods

Mice

Male C57BL/6N and BALB/c mice (9 weeks old) and male ICR mice retired from breeding were purchased from Japan SLC (Hamamatsu, Japan). Mice were housed in a specific pathogen-free and temperature-and humidity-controlled vivarium under a 12-h light, 12-h dark cycle (light on between 0600 and 1800) with free access to chow and water. C57BL/6N and BALB/c mice were housed for at least one week before use in the experiments. All procedures for animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of Kobe University Graduate School of Medicine. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by *the British Journal of Pharmacology*. Especially, to reduce animal suffering, well-trained experimenters continuously monitored the health status of all mice throughout the experiment according to an approved protocol. Mice were euthanized with deep anesthesia using isoflurane or injection of sodium pentobarbital at lethal doses before tissue collection or at the end of experiments, or as soon as if humane endpoints (e.g., deteriorated coat state, continuous eyelid closure, continuous crouching motionless, physical injury) were observed.

Repeated social defeat stress (R-SDS)

R-SDS with two different protocols was used as described previously with minor modifications (McKim et al., 2018; Golden et al., 2011; Nie et al., 2018). Briefly, male ICR mice were screened based on their aggressiveness to a male C57BL/6N mouse, as

measured by the latency and the number of attacks during the observation period (180 s), and were used as aggressor mice for SDS. In R-SDS with Protocol 1, 9-week-old male mice to be defeated and control mice were isolated with free access to chow and water for 1 week. Each of the isolated mice to be defeated was introduced and kept in the home cage of a resident aggressor ICR mouse for 10 min daily for 10 consecutive days. The pairs of defeated and aggressor mice were randomized daily to minimize the variability in the aggressiveness of aggressor mice. Control mice were placed in a novel cage for 10 min daily for 10 consecutive days. In R-SDS with Protocol 2, 9-week-old male mice to be defeated and control mice were group housed (3 mice per cage). An aggressor ICR mouse was introduced into cages of established male cohorts for 6 consecutive days. During each cycle, submissive posture was observed to ensure that the resident mice showed subordinate behavior. If the intruder did not initiate a defeat within 10 min, then a new intruder was introduced. At the end of the 2-h period, the intruder was removed, and the residents were left undisturbed until the next social defeat stress was applied on the following day. Different intruders were used on consecutive days. Control mice were left undisturbed in their home cages. In both Protocol 1 and Protocol 2, aggressive bouts were frequent within first few minutes in each defeat session and were reduced for the remaining period. If an ICR mouse showed over-aggressive behavior, defeated mice were briefly separated to avoid physical injury. As a result, defeated mice rarely received noticeable physical injury. However, a small portion of them were still injured, so that they were euthanized with the methods described above, as soon as humane endpoints described above were observed. As a result, we subjected 187 mice to the experiments and euthanized 13 mice of them because of humane endpoints in the middle of experiments. One mouse was also

removed from subsequent analyses because of the failure of behavioral measurements.

Social interaction test

The social interaction test was performed as previously described (Nie et al., 2018), with minor modifications. A wire-mesh enclosure (10 cm (w) × 6 cm (d); O'Hara, Tokyo, Japan) was placed at one end of a gray plastic open field chamber (42 cm (w) × 42 cm (d) × 40 cm (h); Brain Science Idea, Osaka, Japan). All behavioral tests were conducted under the red light of 10 lux. Mouse behaviors were video-monitored, and the trajectory of mouse ambulation was recorded and automatically determined by a SMART video tracking system (Harvard Apparatus; Holliston, MA, USA). Control and defeated mice were acclimated to the test environment for 1 h before the test. Then, either a naïve or defeated mouse was introduced into the open field chamber without social stimuli for the habituation, and the total distance of ambulation was measured as novelty-induced exploration. After an approximately 1-min interval in the homecage, the mouse was re-introduced into the open field chamber with a novel ICR mouse in the wire-mesh enclosure for the social interaction test. The proportions of times that a mouse spent in the interaction zone (an 8 cm-wide corridor surrounding the wire-mesh enclosure) and the corner zone (two 9 cm × 9 cm squares in the corners of the field opposite the wire-mesh enclosure), respectively, (Figure 1b) in the observation period of 150 s (i.e. “interaction zone %” and “corner zone %” in Figures 1c and 8d) were determined and used as behavioral indices for social interaction and social avoidance, respectively.

Isolation of blood, bone marrow and spleen cells

Blood, bone marrow and spleen cells were isolated as previously described (Katayama

et al., 2006; Engler et al., 2004) with minor modifications. The tissues and blood were collected only once from individual mice. Briefly, mice were deeply anesthetized by inhalation of isoflurane or injection of sodium pentobarbital. Peripheral blood was collected with EDTA-lined syringes from the abdominal portion of vena cava. The counts of white blood cells (WBC) were determined with an automated complete blood count (Nihon Kohden, Tokyo, Japan). Mice were euthanized by deep anesthesia using isoflurane or injection of sodium pentobarbital at lethal doses before tissue collection. Bone marrow was flushed out from the femur with ice-cold RPMI (850 µl; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and the total number of cells was determined using a hemocytometer (MEK-6558, Wakenbtech, Kyoto, Japan). Spleens were collected in ice-cold RPMI, and gently pressed through a 70-µm strainer to obtain a single cell suspension. Erythrocytes were lysed with red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA), and the total number of leukocytes was determined using a hemocytometer. The tissues and blood were collected between 8:00 a.m. and 11:00 a.m.

Flow cytometry

Flow cytometry was performed as previously described (Kawano et al., 2017; Bahr et al., 2018; Evrard et al., 2018). Bone marrow, spleen and blood cells were incubated with anti-CD16/CD32 antibody (clone 93, BioLegend, San Diego, CA, USA; RRID: AB_312801) for Fc receptor block and then incubated with fluorescently labeled antibodies for 15 min at 4°C, as follows: PE-conjugated anti-mouse CD3e (clone 145-2C11, BioLegend; RRID: AB_312672), PerCP-Cy5.5-conjugated anti-mouse CD4 (clone RM-4-5, BioLegend; RRID: AB_893331), FITC-conjugated anti-mouse CD25

(clone PC61.5, Tonbo Biosciences, San Diego, CA, USA; RRID: AB_2621685), PE-conjugated anti-mouse CD45R/B220 (clone RA3-6B2, BioLegend; RRID: AB_312993), APC-conjugated anti-mouse CD45R/B220 (clone RA3-6B2, BioLegend; RRID: AB_312996), PerCP-Cy5.5-conjugated anti-mouse/human CD45R/B220 (clone RA3-6B2, BioLegend; RRID: AB_893356), FITC-conjugated anti-mouse IgM (catalog number 1021-02, Southern Biotechnology, Birmingham, AL, USA; RRID: AB_2794237), FITC-conjugated anti-mouse NK1.1 (clone PK136, BioLegend; RRID: AB_313393), APC-conjugated anti-mouse NK1.1 (clone PK136, BioLegend; RRID: AB_313396), PerCP-Cy5.5-conjugated anti-mouse CD8a (clone 53-6.7, BioLegend; RRID: AB_2075238), PE-conjugated anti-mouse/human CD11b (clone M1/70, BioLegend; RRID: AB_312791), PerCP-Cy5.5-conjugated anti-mouse/human CD11b (clone M1/70, BioLegend; RRID: AB_2129375), PerCP-Cy5.5-conjugated anti-mouse CD115 (clone AFS98, BioLegend; RRID: AB_2566462), APC-conjugated anti-mouse CD115 (clone AFS598, BioLegend; RRID: AB_2085222), APC-conjugated anti-mouse Ly6C (clone HK1.4, BioLegend; RRID: AB_1732076), APC-conjugated anti-mouse F4/80 (clone BM8, BioLegend; RRID: AB_893481), FITC-conjugated anti-mouse Gr-1 (clone RB6-8C5, Tonbo Biosciences; RRID:AB_2621721), PerCP-Cy5.5-conjugated anti-mouse Gr-1 (clone RB6-8C5, BioLegend; RRID: AB_893561), APC-conjugated anti-mouse CD90.2 (clone 53-2.1, BioLegend; RRID: AB_10645337), APC-conjugated anti-mouse I-A/I-E (clone M5/114.15.2, BioLegend; RRID: AB_313328), APC-Cy7-conjugated anti-mouse Siglec-F (clone E50-2440, BD Biosciences; RRID: AB_2732831), APC-conjugated anti-mouse CD117 (cKit) (clone 2B8, BioLegend; RRID: AB_313220), APC-conjugated anti-mouse CXCR4 (clone L276F12, BioLegend; RRID: AB_2562784), BV421-conjugated anti-mouse CXCR4 (clone L276F12,

BioLegend; RRID: AB_2562788), FITC-conjugated anti-mouse Ly6G (clone 1A8, BioLegend; RRID: AB_1236494), BV421-conjugated anti-mouse Ly6G (clone 1A8, BioLegend; RRID: AB_2562567), PE-Cy7-conjugated anti-mouse CD101 (clone Moushi101, eBioscience; RRID: AB_2573378), APC-conjugated anti-mouse CD45 (clone 30-F11, BioLegend; RRID: AB_312977), PE-conjugated anti-mouse CCR2 (clone SA203G11, BioLegend; RRID: AB_2616981), PerCP-Cy5.5-conjugated anti-mouse TER-119/Erythroid Cells (clone TER-119, BioLegend; RRID: AB_893638), BV421-conjugated anti-mouse Sca-1 (clone D7, BioLegend; RRID: AB_10898327), PE-conjugated anti-mouse CD127 (IL-7Rα) (clone A7R34, BioLegend; RRID: AB_1937251), APC-Cy7-conjugated anti-mouse CD16/32 (clone 93, BioLegend; RRID: AB_1967102), FITC-conjugated anti-mouse CD34 (clone RAM34, eBioscience; RRID: AB_465021). Nonspecific binding was assessed using isotype-matched or appropriate control antibodies. Fluorescent signals were determined using Accuri C6 (BD Biosciences, San Jose, CA, USA) and CytoFLEX S (Beckman Coulter, Brea, CA, USA).

Data were analyzed using FlowJo software (FlowJo LLC, BD Biosciences; RRID: SCR_008520) with gating strategies shown in Supplemental Figures 1-9. We used forward versus side scatter plots to gate leukocytes, as previously described (e.g., Kawano et al., 2017). Virtually all (>99%) leukocytes identified by this gating strategy were stained by CD45, a typical marker for entire leukocytes. Multiple sets of markers have been used to define some leukocyte subsets. For example, mature and immature neutrophils were defined as $CD11b^{+}F4/80^{-}Gr\text{-}1^{hi}$ and $CD11b^{+}F4/80^{-}Gr\text{-}1^{lo}$ leukocytes, respectively, as previously confirmed with Giemsa stain (Kawano et al., 2017). We compared these subsets with the distribution of Ly6G, another typical marker for

neutrophils by quadruple staining of CD11b, F4/80, Gr-1 and Ly6G. Although $CD11b^{+}F4/80^{-}Gr\text{-}1^{hi}$ cells were almost (>98%) Ly6G positive in the bone marrow and blood, $CD11b^{+}F4/80^{-}Gr\text{-}1^{lo}$ cells only partially expressed Ly6G in the bone marrow. Thus, we analyzed immature and mature neutrophils using different sets of markers (i.e. $Lin^{-}cKit^{-}CXCR4^{-}CD11b^{+}Gr\text{-}1^{+}CD101^{-}$ and $Lin^{-}cKit^{-}CXCR4^{-}CD11b^{+}Gr\text{-}1^{+}CD101^{+}$, respectively; e.g., Evrard et al., 2018) and confirmed that the same results were obtained with these different sets of markers (see Results). As another example, NK cells were defined as $NK1.1^{+}CD3e^{-}$ cells, as previously described (Jiang et al., 2017). We compared this subset with the distribution of CD49b, another typical marker for pan-NK cells, and found that $NK1.1^{+}CD3e^{-}$ cells are virtually identical (>98%) for $CD49b^{+}$ NK cells.

Measurement of chemokine concentrations in the bone marrow and blood

The concentrations of chemokines were measured, as previously described with minor modifications (Katayama et al., 2006). Briefly, mice were deeply anesthetized by injection of sodium pentobarbital. Peripheral blood was collected by inserting a needle to the heart. The whole blood was kept at room temperature for 2 h to form blood clots. The supernatant was harvested after centrifugation at 2000 × g for 20 min and used as the serum. After the mice were euthanized by deep anesthesia using injection of sodium pentobarbital at lethal doses, a femur was obtained and flushed with 400 µl of ice-cold PBS. The supernatant was harvested after centrifugation at 400 × g for 5 min and used as the bone marrow extracellular fluid (BMEF). The concentrations of C-C motif chemokine 2 (CCL2) and C-X-C motif chemokine 12 (CXCL12) in the serum and BMEF were measured using the Quantikine ELISA kits (#MJE00B and #MCX120,

R&D Systems, MN, USA) according to the manufacturer's instructions.

Data and statistical analysis

The data and statistical analysis comply with the recommendations made by *the British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). For all animal experiments, at least five mice were included in each group, and at least two repeat experiments were carried out. Experiments were designed to make sample sizes relatively equal and randomized among comparison groups. Sample sizes were determined according to previous studies with similar analyses. Same parameters for leukocyte and behavioral analyses were unbiasedly applied among comparison groups. The number of independent values (without treating technical replicates as independent values) was declared and used for statistical analyses. All data points were included for statistical analyses. Values without normalization were used for statistical analyses.

Data are presented as mean values ± SEM. Statistical significance was determined using unpaired *t* test in Figure 2a, 2c, 3, 4, 5, 6, 7, Supplemental Figure 11. Comparisons among multiple groups were performed with two-way repeated measures ANOVA in Figures 1 and 8d (Test Condition as a within-subjects variable); with two-way ANOVA in Figures 2b, 9, and 10; with three-way repeated measures ANOVA in Figure 8b (Day as a within-subjects variable); and with one-way ANOVA in Supplemental Figure 10. For these analyses, post hoc pairwise comparisons were performed using Tukey's multiple comparison test (or Bonferroni's multiple comparison test, if Tukey's test was not applicable), only if a main effect for at least one factor or the interaction between two factors showed statistical significance. $P < 0.05$ was considered significant.

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA; RRID: SCR_002798).

Results

Repeated social defeat stress increases neutrophils and monocytes and decreases lymphocytes in the bone marrow of C57BL/6N mice.

We examined the behavioral effects of R-SDS with Protocol 1 and Protocol 2 (see Introduction; Figures 1a). Control mice in Protocol 1 appeared to show social interaction with an ICR mouse, whereas those in Protocol 2 did not, as seen in longer durations spent in the interaction zone during the social interaction test than during the habituation. This could be due to the difference in housing conditions (i.e. single-housed in Protocol 1 and group-housed in Protocol 2). By contrast, defeated mice regardless of the two protocols showed a reduced time for the interaction zone and an increased time for the avoidance zone only in the presence of an ICR mouse, demonstrating R-SDS-induced social avoidance. Locomotor activity without an ICR mouse was not affected with either protocol (Figures 1b-d; e.g., $P < 0.05$ for the Stress × Test Condition interaction for the interaction and corner zones in two-way repeated-measures ANOVA).

Then we compared the effects of R-SDS with the two protocols on the numbers of leukocyte subsets in the bone marrow of C57BL/6N mice. Whereas the total number of bone marrow cells was not changed, R-SDS with both protocols decreased the numbers of lymphocytes (i.e. total T cells ($CD3e^+$), helper T cells ($CD3e^+CD4^+$), regulatory T cells ($CD3e^+CD4^+CD25^+$), cytotoxic T cells ($CD3e^+CD8a^+$), total B cells ($B220^+$), mature B cells ($B220^+IgM^+$), immature B cells ($B220^+IgM^-$) and NK cells ($NK1.1^+CD3e^-$)) immediately after the last stress exposure (Figures 2a, c, Supplemental Table 1). R-SDS with both protocols also decreased the number of $CD11c^+CD11b^-$ dendritic cells (Figures 2a, c). By contrast, the numbers of monocytes

($CD11b^+CD115^+$), especially $Ly6C^{hi}$ monocytes ($CD11b^+CD115^+Ly6C^{hi}$) and neutrophils ($CD11b^+F4/80^-Gr\text{-}1^+$), were increased with both protocols (Figures 2a, c). These findings show that R-SDS increases the numbers of neutrophils and monocytes and decreases the numbers of lymphocytes in the bone marrow, regardless of the stress protocols.

Notably, in Protocol 1, the increase in mature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{hi}$) occurred only after the 10th stress exposure (Figure 2a), thus later than the increase in immature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{lo}$) that appeared as early as after the 4th stress exposure (Figure 2b; $P < 0.05$ for the Stress and Day main effects, and the Stress × Day interaction in two-way ANOVA). In Protocol 2 with fewer stress exposure than Protocol 1, the number of immature neutrophils, but not of mature neutrophils, was increased (Figure 2c). Note that these results were confirmed with different sets of markers (i.e. $Lin^-cKit^-CXCR4^-CD11b^+Gr\text{-}1^+CD101^-$ and $Lin^-cKit^-CXCR4^-CD11b^+Gr\text{-}1^+CD101^+$, respectively; Supplemental Figure 10). We also examined the number of myeloid and lymphoid progenitors in the bone marrow. The numbers of LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) and granulocyte/monocyte progenitors were not changed, whereas these of common lymphoid progenitors, common myeloid progenitors and megakaryocyte/erythrocyte progenitors were decreased after R-SDS (Supplemental Figure 11). These findings suggest that R-SDS specifically increases the number of immature neutrophils, which become mature after a certain period with the stress exposure.

Repeated social defeat stress increases neutrophils and $Ly6C^{hi}$ monocytes in the blood and spleen of C57BL/6N mice.

We next compared the effects of R-SDS with the two protocols (Figures 1a) on the

numbers of leukocyte subsets in the blood of C57BL/6N mice. R-SDS decreased the numbers of T cells, B cells, NK cells and dendritic cells as well as the total number of white blood cells immediately after the last stress exposure with Protocol 2, but not with Protocol 1 (Figures 3a, b, Supplemental Table 2). The numbers of $Ly6C^{hi}$ monocytes and neutrophils were increased with both protocols, whereas that of $Ly6C^{lo}$ monocytes ($CD11b^{+}CD115^{+}Ly6C^{lo}$) was decreased only with Protocol 2 (Figures 3a, b). These findings show that R-SDS increases the numbers of neutrophils and $Ly6C^{hi}$ monocytes in the blood of C57BL/6N mice, regardless of the stress protocols. We also examined the effects of R-SDS with Protocol 2 on the numbers of monocytes and neutrophils in the spleen, another source of circulating monocytes and neutrophils (Bronte et al., 2013) (Figure 4a, Supplemental Table 3). R-SDS increased $Ly6C^{hi}$ monocytes and mature neutrophils in the spleen (Figures 4b-d).

Repeated social defeat stress alters CCL2-CCR2 and CXCL12-CXCR4 pathways in the bone marrow and blood of C57BL/6N mice.

CCL2-CCR2 (C-C chemokine receptor type 2) and CXCL12-CXCR4 (C-X-C chemokine receptor type 4) pathways are key regulators for the mobilization of monocytes and neutrophils. To determine whether R-SDS alters these pathways in the bone marrow and blood, CCL2 and CXCL12 were examined in the BMEF and serum. R-SDS increased the levels of CCL2 in the BMEF and serum (Figures 5a, 5d). The level of CXCL12 was decreased in BMEF, whereas it was slightly increased in the serum (Figures 6a, 6d). We also examined the expressions of CCR2 on monocytes and CXCR4 on neutrophils in the bone marrow and blood. R-SDS decreased the mean fluorescent intensity (MFI) of CCR2 on total monocytes, $Ly6C^{hi}$ monocytes and $Ly6C^{lo}$ monocytes

in the bone marrow and blood as well as the proportion of monocytes with detectable CCR2 expression in the bone marrow (Figures 5b, 5c, 5e and 5f). The proportion of cells with detectable CXCR4 expression were decreased in bone marrow neutrophils (Figures 6b, 6c, 6e and 6f). These findings show that R-SDS alters CCL2-CCR2 and CXCL12-CXCR4 pathways in the bone marrow and blood, suggesting their roles for R-SDS-induced mobilization of monocytes and neutrophils.

Repeated social defeat stress-induced neutrophil increase is maintained after the stress in the bone marrow and blood of C57BL/6N mice.

To determine whether the stress-induced changes in the bone marrow and blood were maintained after the last stress, we examined the effects of R-SDS with Protocol 2 on the numbers of leukocyte subsets at 6 days after the last stress exposure (Figure 7a, Supplemental Table 4). In the bone marrow, the total cell number was unaltered, as observed immediately after the last stress exposure. R-SDS-induced changes in the numbers of T cells, B cells and monocytes significantly remained, but became smaller for 6 days after the last stress exposure (Figures 7b; see Figures 2c). R-SDS-induced increase in the number of neutrophils was maintained to a level similar to that immediately after the last stress exposure (Figure 7b). Notably, whereas only immature neutrophils showed an increase immediately after the last stress exposure, as described above (see Figure 2c), the numbers of both immature and mature neutrophils were increased at 6 days after the last stress exposure (Figure 7b). This finding suggests that the number of immature neutrophils continues to increase after cessation of stress. In the blood, the total cell number was unaltered (Figure 7c). Whereas R-SDS-induced changes in the numbers of T cells, B cells and monocytes greatly diminished for 6 days

after the last stress exposure, R-SDS-induced increase in the number of circulating neutrophils was still noticeable (Figure 7c).

Repeated social defeat stress-induced increase in circulating neutrophils is larger in BALB/c mice than in C57BL/6N mice.

It has been shown that BALB/c mice are more susceptible to repeated social defeat stress and other types of chronic stress than C57BL/6N mice (Razzoli et al., 2011; Uchida et al., 2011; Laine et al., 2018). To examine whether R-SDS-induced hematological changes account for the strain-dependent difference in stress susceptibility, we compared the effects of R-SDS with Protocol 2 on the numbers of leukocyte subsets in the bone marrow and blood of C57BL/6N and BALB/c mice (Figure 8a). As previously reported (Laine et al., 2018), R-SDS induced the loss of the body weight only in BALB/c mice (Figure 8b, P <0.05 for the Stress, Strain and Day main effects, and the Stress × Strain × Day interaction in three-way repeated measures ANOVA). In mice of both strains, whereas locomotor activity in a novel environment was not affected (Figure 8c), R-SDS induced social avoidance from a novel ICR mouse in the social interaction test (Figure 8d; $P < 0.05$ for the Test Condition main effect in two-way repeated measures ANOVA for the interaction and corner zones). Notably, R-SDS-induced social avoidance was larger in BALB/c mice than in C57BL/6N mice, suggesting higher stress susceptibility in BALB/c mice, as previously reported (Figure 8d; $P < 0.05$ for the Test Condition × Strain interaction in two-way repeated measures ANOVA for the corner zone). In the bone marrow, R-SDS did not affect the total number of leukocytes in both strains (Figure 9, Supplemental Table 5). Bone marrow T cells and their subsets were fewer in BALB/c mice than in C57BL/6N mice without R-

SDS, and failed to show R-SDS-induced decrease in BALB/c mice that was observed in C57BL/6N mice (Figure 9; e.g., $P < 0.05$ for the Stress × Strain interaction for T cells in two-way ANOVA). This finding suggests that R-SDS-induced change in the number of bone marrow T cells is not involved in the induction of social avoidance. By contrast, both strains showed R-SDS-induced decrease in the number of B cells and increase in the number of neutrophils in the bone marrow, although bone marrow neutrophils and B cells, especially immature neutrophils and IgM^+ B cells, without R-SDS were significantly fewer in BALB/c mice than in C57BL/6N mice (Figure 9; e.g., $P < 0.05$ for the Stress and Strain main effects for B cells and total and immature neutrophils in two-way ANOVA). These findings show that R-SDS increases the number of neutrophils and decreases the number of B cells in the bone marrow of both C57BL/6N and BALB/c mice, although these changes cannot account for the difference in stress susceptibility between the two strains.

In the blood, R-SDS did not affect the number of white blood cells in either strain (Figure 10, Supplemental Table 6). R-SDS-induced decrease in the numbers of T cells and most of their subsets was observed in C57BL/6N mice, but not in BALB/c mice (Figure 10; e.g., $P < 0.05$ for the Strain main effect for total T cells and helper T cells and for the Stress × Strain interaction for cytotoxic T cells in two-way ANOVA), suggesting that this change in the number of T cells is not involved in the induction of social avoidance. R-SDS decreased the numbers of total B cells and IgM^+ B cells in both strains, although these cells were fewer in BALB/c mice than in C57BL/6N mice (Figure 10, $P < 0.05$ for the Stress and Strain main effects in two-way ANOVA). Neutrophils showed R-SDS-induced increase in number in both strains (Figure 10, $P < 0.05$ for the Stress and Strain main effects in two-way ANOVA). Notably, this increase

was also significantly larger in BALB/c mice than in C57BL/6N mice. Stress susceptibility also varies even within mice of the same strain (see Figure 8). Defeated mice which showed social avoidance or those which did not are frequently categorized to susceptible or resilient mice, respectively. In exploratory analyses, the numbers of neutrophils and B cells in the bone marrow and blood were not different between susceptible and resilient mice of either strain (Supplemental Figure 12). These findings show that R-SDS decreases B cells and increases neutrophils to different extents in the blood of the two strains with different stress susceptibility, although these changes did not represent individual variability of stress susceptibility in the same genetic backgrounds.

Discussion

Here we showed that R-SDS increased neutrophils and monocytes and decreased T cells, B cells, NK cells and dendritic cells in the bone marrow of C57BL/6N mice with two protocols with different stress conditions. These changes in neutrophils, monocytes, and T and B cells were maintained for at least 6 days after R-SDS. In the blood, R-SDS increased neutrophils and monocytes with both protocols, whereas lymphocytes and dendritic cells were decreased with only one of these. Notably, the increase in circulating neutrophils rather than monocytes was maintained for 6 days after R-SDS. Furthermore, R-SDS-induced increase in circulating neutrophils was larger in BALB/c mice than in C57BL/6N mice, the former of which are more susceptible to R-SDS than the latter. These findings show that R-SDS induces neutrophil mobilization and its maintenance, and suggest that these neutrophil changes are related to genetic variability of stress susceptibility.

Mechanisms of stress-induced leukocyte mobilization

Since the increase in neutrophils rather than monocytes in the blood was maintained for at least 6 days after R-SDS, different mechanisms could be used to induce the mobilization of these cell types. Previous studies have indicated that various stressors activate sympathetic nerves projecting to the bone marrow (Haffner-Luntzer et al., 2019; Heidt et al., 2014). It has also been suggested that sympathetic activation in the bone marrow induces the mobilization of monocytes and neutrophils from the bone marrow through regulating CCL2-CCR2 and CXCL12-CXCR4 pathways, respectively (Shi et al., 2011; De Filippo et al., 2018). CCL2-CCR2 pathway augments the mobilization of monocytes from the bone marrow. We found that R-SDS increased the

level of CCL2 and decreased the expression of CCR2 in monocytes in the bone marrow and blood. Given that the surface expression of CCR2 is downregulated upon its activation, this finding suggests that R-SDS activates CCL2-CCR2 pathway in bone marrow and circulating monocytes. CXCL12-CXCR4 pathway rather retains neutrophils in the bone marrow, so that its inhibition augments the mobilization of neutrophils from the bone marrow. R-SDS decreased CXCL12 production and neutrophil CXCR4 expression in the bone marrow, suggesting the downregulation of CXCL12-CXCR4 pathway in bone marrow neutrophils. Thus, the upregulation of CCL2-CCR2 pathway in monocytes and the downregulation of CXCL12-CXCR4 pathway in bone marrow neutrophils could contribute to R-SDS-induced mobilization of the respective cells.

In addition, John Sheridan and his colleagues have shown that glucocorticoid is crucial for R-SDS-induced increase in circulating monocytes (Niraula et al., 2018). In their study, glucocorticoid depletion appears to spare R-SDS-induced increase in circulating neutrophils (c.f. Figures 1G and 4I in Niraula et al., 2018). G-CSF is a cytokine that plays crucial roles in neutrophil expansion and mobilization (Semerad et al., 2002). Several cytokines, especially IL-17, increases blood G-CSF, leading to neutrophil expansion and mobilization (Stark et al., 2005). It has been reported that both IL-17 and G-CSF in the blood are increased by repeated electric footshock stress (Cheng et al., 2015). Repeated restraint stress also increases Th17 cells, which secretes IL-17, in the brain (Beurel et al., 2013). Previous studies using genetic and pharmacological manipulations of Th17 cells and IL-17 have shown that IL-17 is crucial for stress-induced depressive-like behavior in rodents (Beurel et al., 2013; Nadeem et al., 2017). Clinical studies have reported that depressive patients show the

increase in neutrophils, IL-17 and G-CSF in the blood (Lynall et al., 2020; Syed et al., 2018). Thus, IL-17-G-CSF pathway could mediate stress-induced neutrophil mobilization, thereby promoting stress-related pathology of depression. Note that the possibility that the redistribution of neutrophils among different immune compartments also contributes to stress-induced neutrophil increase cannot be excluded so far.

By contrast, how R-SDS-induced neutrophil mobilization is maintained remains elusive. One possibility is that R-SDS-induced increase in G-CSF and other cytokines that preferentially affect neutrophils is maintained after the stress. Alternatively, but not exclusively, R-SDS could alter the properties of neutrophil progenitors and/or stromal and endothelial cells, and this alteration could be maintained after the stress. Since R-SDS mobilizes hematopoietic progenitors from the bone marrow to the spleen and promotes splenic myelopoiesis (McKim et al., 2018), the spleen may also contribute to the maintenance of R-SDS-induced neutrophil increase. This possibility is corroborated by the fact that Angiotensin II and its type 1 receptor are involved in neutrophil and monocyte mobilization from the spleen (Mellak et al., 2015) and in R-SDS-induced cardiovascular alterations (Brouillard et al., 2019). However, in our experimental condition, R-SDS increased mature neutrophils in the spleen, but the increase was less evident in immature neutrophils, suggesting that the neutrophil increase in the spleen shortly after R-SDS results from the homing/lodgment of circulating neutrophils.

Functional and clinical implications of stress-induced leukocyte changes

As described above, the findings in this study indicate the association of neutrophils rather than monocytes in the blood with R-SDS-induced social avoidance. Previous studies in rodent stress models have claimed the behavioral importance of monocytes,

based on the findings that genetic deletion of CCR2 abolished both stress-induced brain infiltration of monocytes and behavioral anxiety (Wohleb et al., 2013). However, not only monocytes but also neutrophils can secrete IL-1β and IL-6 (Tecchio et al., 2014), which underlie R-SDS-induced behavioral changes (McKim et al., 2018; Hodes et al., 2014; Menard et al., 2017) and neutrophils outnumbered monocytes after R-SDS in this study. Furthermore, neutrophils and monocytes can interact with each other for reciprocal activation through cytokine secretion (Mantovani, et al., 2011, Prame Kumar et al., 2018). Roles of neutrophils in depressive-like behavior are emerging, as treatment of lipopolysaccharide promotes the migration of neutrophils into the brain and causes depressive-like behaviors (Aguilar-Valles et al., 2014).

Although the cumulative stress duration was greater in Protocol 2 than in Protocol 1, defeated mice derived from the two protocols showed comparable levels of social avoidance. However, R-SDS with Protocol 2, but not with Protocol 1, decreased the numbers of lymphocytes and dendritic cells in the blood, although R-SDS with the two protocols similarly decreases these cells in the bone marrow. Thus, there may be a dichotomy between the behavioral and immunological effects of chronic stress. Although chronic stress has been associated with reduced cellular and humoral immune responses (Segerstrom and Miller, 2004), how stress conditions affect stress-induced immune suppression remains elusive. The two R-SDS protocols used in this study have multiple differences, such as the stress duration of each cycle (10 min or 2 h), the number of stress cycles (10 or 6 days), the pattern of attacks including biting, the housing condition (single- or group-housed), and a stress environment (familiar or unfamiliar cage). It was reported that defeated mice with bites showed larger increase in myeloid cells and decrease in lymphoid cells than those without bites (Engler et al.,

2004), although repeated stress without bites (e.g., restraint stress) induces similar leukocyte changes (Tournier et al., 2001; Jiang et al., 2017). Since prolonged social isolation alone induces depressive-like behaviors (Ieraci et al., 2016), single housing in Protocol 1 could augment the effect of R-SDS. The familiarity of stress environments could also alter the patterns of defensive behaviors to stressors (Mongeau et al., 2003). Systematic behavioral analyses are necessary to elucidate the behavioral and immunological contributions of these stress variables.

The interaction between genetic and environmental factors is crucial for the pathology of mental illnesses. Since R-SDS increased neutrophils and decreased B cells to different extents in the blood of the two mouse strains with different stress susceptibility, but similarly in susceptible and resilient mice, these leukocyte changes could represent genetic variability of stress susceptibility and mediate gene-environment interaction in stress-related pathology. Clinical studies have reported an increase in circulating neutrophils in depressive patients in a manner correlated to the severity of depressive symptoms (e.g., Lynall et al., 2020; Özyurt et al., 2018). Given the association of circulating neutrophils and stress-induced behavioral changes in this study, it is intriguing to test whether neutrophil increase in depressive patients reflects the state of stress and contributes to depressive symptoms. Preclinical studies in rodents have shown that prostaglandin E_2, TNF-α and IL-6 are crucial for stress-induced depressive-like behaviors, and promote clinical trials with these inhibitors (Müller et al., 2010; Brymer et al., 2019; Yi-Chih Ting et al., 2020). Stress-mobilized neutrophils may be responsible for secretion of these inflammation-related molecules to promote depression pathology. This study may add cytokines and chemokines responsible for stress-induced neutrophil mobilization, as discussed above, in the list of therapeutic

targets for depression. Thus, this line of research paves the way for understanding stress-related pathology and promoting therapeutic development in mental illnesses.

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Conflict of interests

Yuka Ishikawa and Taro Kato are employees of Sumitomo Dainippon Pharma Co., Ltd.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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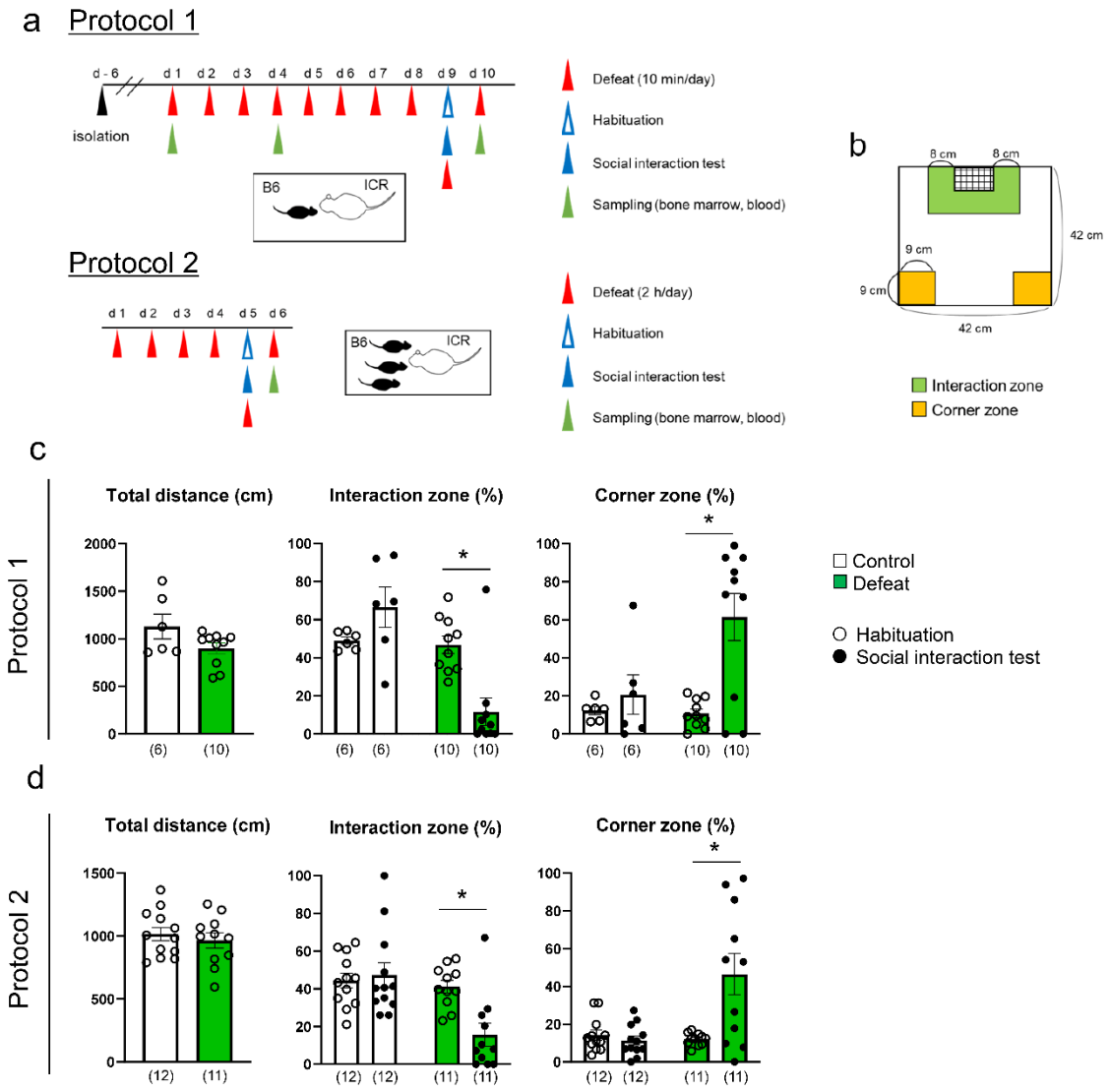


Figure 1. R-SDS with two different protocols induced social avoidance. (**a**) Two different protocols of R-SDS. In Protocol 1, a mouse to be defeated is single-housed and exposed to an ICR aggressor mouse for 10 min daily for 10 consecutive days. In Protocol 2, a mouse to be defeated is group-housed and exposed to an ICR aggressor mouse for 2 h daily for 6 consecutive days. The mouse received the social interaction test as well as the habituation to its experimental chamber before the stress one day before the last stress (d9 for Protocol 1 and d5 for Protocol 2). Cells in the bone marrow and blood were collected immediately after the 1st, 4th and 10th stress for Protocol 1 and

after the 6th stress for Protocol 2. (b) The definitions of the interaction zone and the corner zone. (c, d) Novelty-induced exploration and social avoidance without or with R-SDS (open and green bars, respectively) with Protocol 1 (c) and Protocol 2 (d). Novelty-induced exploration was measured as the total distance of ambulation during the habituation before the social interaction test. The proportions of the times spent in the interaction zone and the corner zone during the social interaction test and its habituation were measured to assess the levels of social interaction and avoidance, respectively. The number of mice is shown below each bar. Bars represent the mean ± SEM. *$P < 0.05$ for two-way repeated ANOVA followed by Bonferroni's post-hoc test.

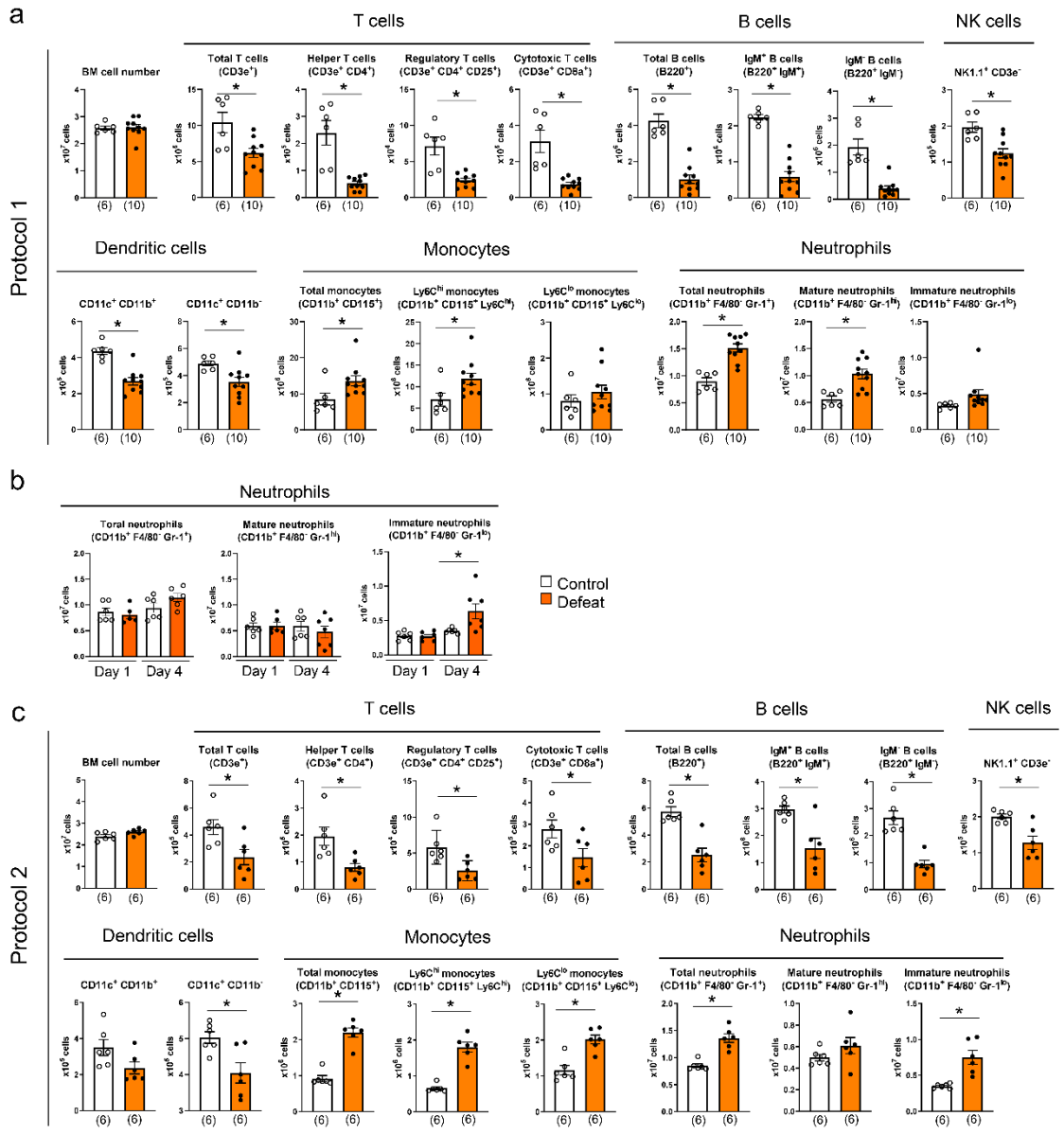


Figure 2. R-SDS increased neutrophils and monocytes, whereas decreased T cells, B cells, NK cells and dendritic cells, in the bone marrow of C57BL/6N mice. (**a-c**) R-SDS-induced changes in the cell numbers of distinct leukocyte subsets in the bone marrow of each femur with Protocol 1 (**a**, **b**) and Protocol 2 (**c**). Cells in the bone marrow were collected immediately after the 1st, 4th and 10th stress for Protocol 1 and after the 6th stress for Protocol 2. The numbers of the following subsets without R-SDS

(open bars) or after the last stress of R-SDS (orange bars) are shown: bone marrow (BM) cells, total T cells ($CD3e^+$), helper T cells ($CD3e^+CD4^+$), regulatory T cells ($CD3e^+CD4^+CD25^+$), cytotoxic T cells ($CD3e^+CD8^+$), total B cells ($B220^+$), mature B cells ($B220^+IgM^+$), immature B cells ($B220^+IgM^-$), NK cells ($NK1.1^+CD3e^-$), dendritic cells ($CD11c^+CD11b^+$ and $CD11c^+CD11b^-$), total monocytes ($CD11b^+CD115^+$), $Ly6C^{hi}$ monocytes ($CD11b^+CD115^+Ly6C^{hi}$), $Ly6C^{lo}$ monocytes ($CD11b^+CD115^+Ly6C^{lo}$), total neutrophils ($CD11b^+F4/80^-Gr\text{-}1^+$), mature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{hi}$), and immature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{lo}$). Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. *$P < 0.05$ for unpaired *t* test (**a**, **c**) or two-way ANOVA followed by Tukey's post-hoc test (**b**).

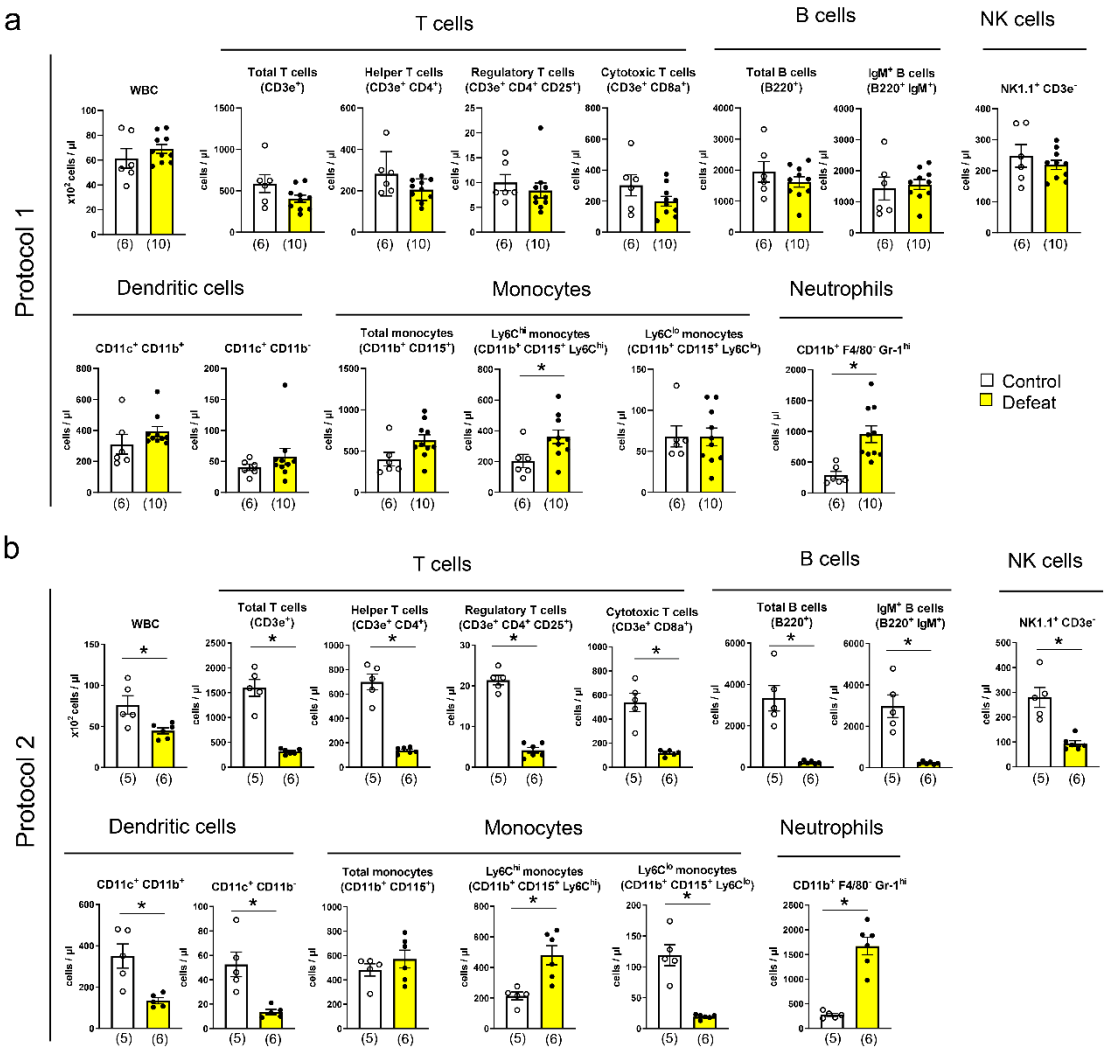


Figure 3. R-SDS increased neutrophils and Ly6C^{hi} monocytes in the blood of C57BL/6N mice. The effects of R-SDS with Protocol 1 (**a**) and Protocol 2 (**b**) were analyzed. Cells in the blood were collected immediately after the 10th stress for Protocol 1 and after the 6th stress for Protocol 2. The numbers of the following subsets without R-SDS (open bars) or after the last stress of R-SDS (yellow bars) are shown: white blood cells (WBC), total T cells (CD3e$^+$), helper T cells (CD3e$^+$CD4$^+$), regulatory T cells (CD3e$^+$CD4$^+$CD25$^+$), cytotoxic T cells (CD3e$^+$CD8$^+$), total B cells (B220$^+$), IgM-positive B cells (B220$^+$IgM$^+$), NK cells (NK1.1$^+$CD3e$^-$), dendritic cells (CD11c$^+$CD11b$^+$ and CD11c$^+$CD11b$^-$), total monocytes (CD11b$^+$CD115$^+$), Ly6C^{hi} monocytes

($CD11b^+CD115^+Ly6C^{hi}$), $Ly6C^{lo}$ monocytes ($CD11b^+CD115^+Ly6C^{lo}$), and mature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{hi}$). Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. *$P <$ 0.05 for unpaired *t* test.

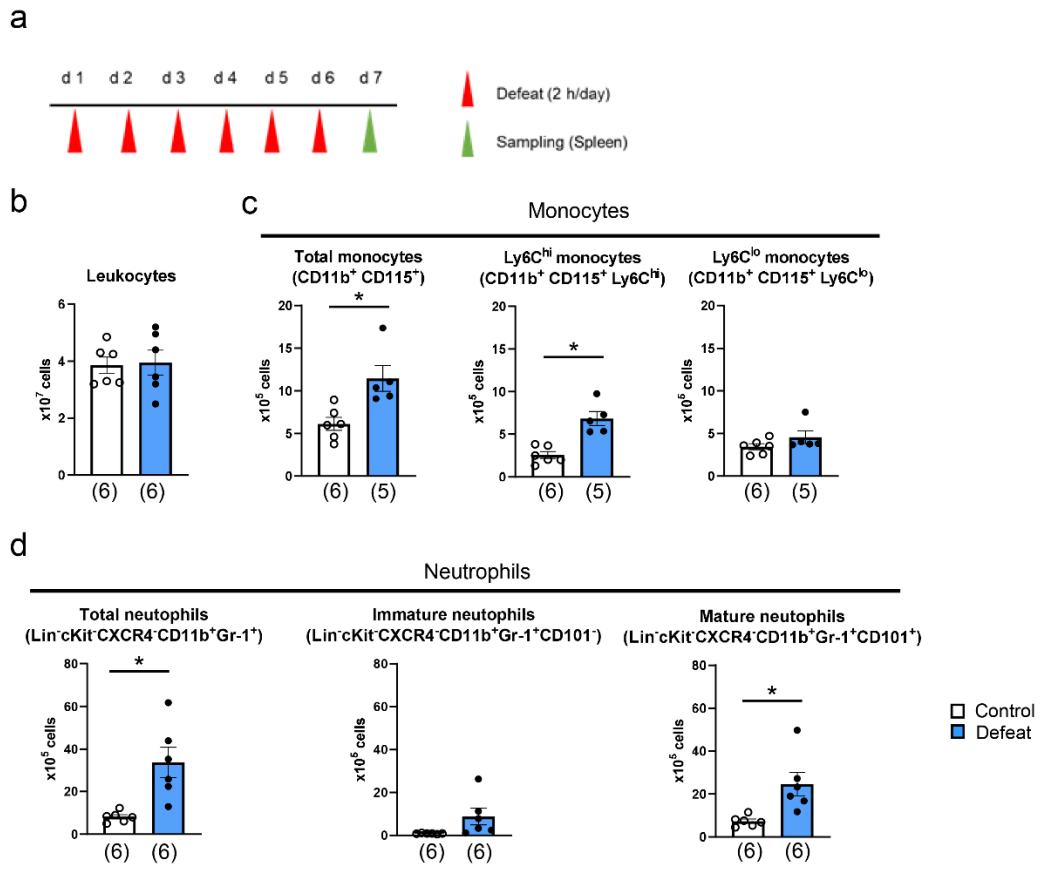


Figure 4. R-SDS increased neutrophils and monocytes in the spleen of C57BL/6N mice. (**a**) An experimental schedule. R-SDS with Protocol 2 is used. A mouse to be defeated is group-housed and exposed to an ICR aggressor mouse for 2 h daily for 6 consecutive days. Spleens were collected at 12-14 h after the last stress (d7). (**b-d**)

The numbers of the following subsets in the spleen without (open bars) or with (blue bars) R-SDS are shown: total leukocytes in (**b**), total monocytes ($CD11b^{+}CD115^{+}$), $Ly6C^{hi}$ monocytes ($CD11b^{+}CD115^{+}Ly6C^{hi}$) and $Ly6C^{lo}$ monocytes ($CD11b^{+}CD115^{+}Ly6C^{lo}$) in (**c**), and total neutrophils ($Lin^{-}cKit^{-}CXCR4^{-}CD11b^{+}Gr\text{-}1^{+}$), immature neutrophils ($Lin^{-}cKit^{-}CXCR4^{-}CD11b^{+}Gr\text{-}1^{+}CD101^{-}$) and mature neutrophils ($Lin^{-}cKit^{-}CXCR4^{-}CD11b^{+}Gr\text{-}1^{+}CD101^{+}$) in (**d**). Lineage markers (Lin) used for gating neutrophils are B220, NK1.1, CD90.2, CD115, Siglec-F, and MHCII. Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. $*P < 0.05$ for unpaired *t* test.

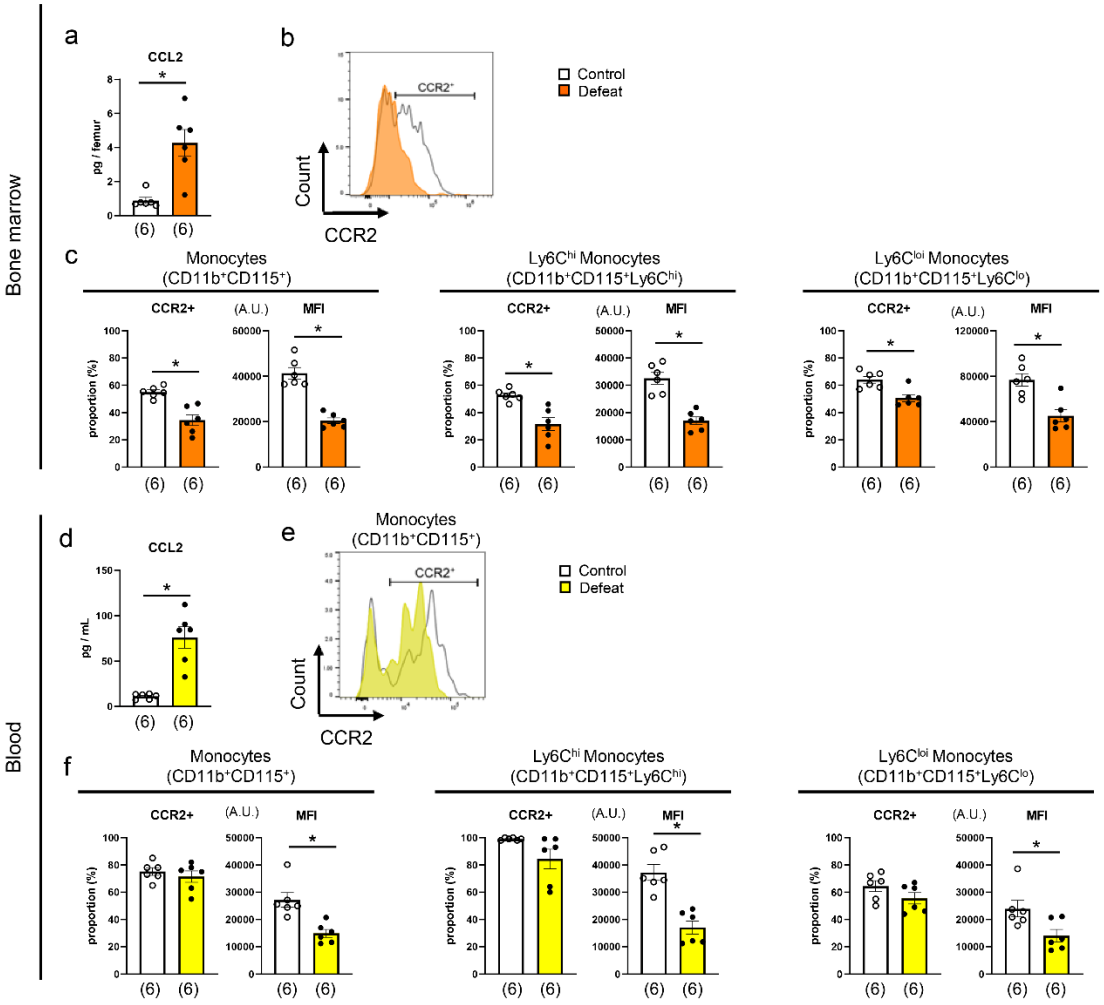


Figure 5. R-SDS altered CCL2 levels and monocyte CCR2 expression in the bone marrow and blood of C57BL/6N mice. The effects of R-SDS with Protocol 2 were analyzed. Cells in the bone marrow were collected at 12-14 h after the last stress. CCL2 levels in the BMEF (**a**) and serum (**d**), and the proportions of cells with detectable CCR2 expression in total monocytes ($CD11b^{+}CD115^{+}$), $Ly6C^{hi}$ monocytes ($CD11b^{+}CD115^{+}Ly6C^{hi}$) and $Ly6C^{lo}$ monocytes ($CD11b^{+}CD115^{+}Ly6C^{lo}$) and the MFI in the bone marrow (**c**) and blood (**f**), without or with R-SDS (open or orange bars for the bone marrow; open or yellow bars for the blood) are shown. Representative histograms

of CCR2 expression on total monocytes in the bone marrow (**b**) and blood (**e**) are also shown. Note that some data points in CCL2 measurements in the BMEF were below the concentration range used to generate the standard curve. Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. **P* < 0.05 for unpaired *t* test.

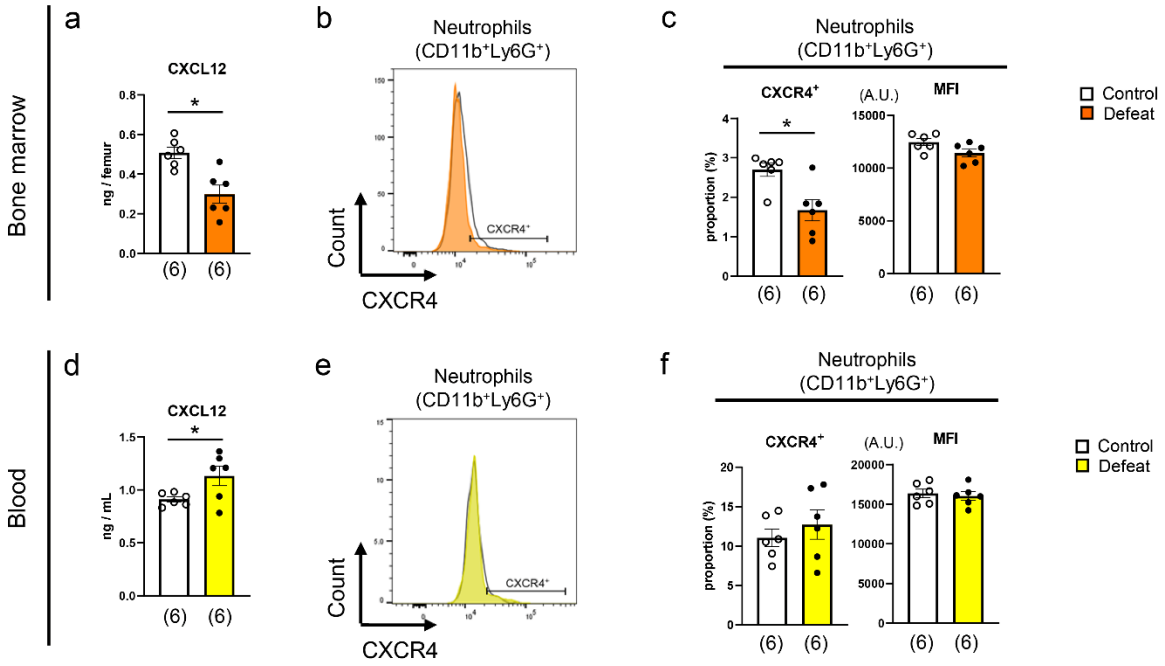


Figure 6. R-SDS altered CXCL12 levels and neutrophil CXCR4 expression in the bone marrow and blood of C57BL/6N mice. The effects of R-SDS with Protocol 2 were analyzed. Cells in the bone marrow were collected at 12-14 h after the last stress. CXCL12 levels in the BMEF (**a**) and serum (**d**), and the proportions of cells with detectable CXCR4 expression in neutrophils ($CD11b^+Ly6G^+$) and the MFI in the bone marrow (**c**) and blood (**f**) without or with R-SDS (open or orange bars for the bone marrow; open or yellow bars for the blood) are shown. Representative histograms of CXCR4 expression on neutrophils

in the bone marrow (**b**) and blood (**e**) are also shown. The histogram of CXCR4-expressing cells is amplified in an inset in (**e**). Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. **P* < 0.05 for unpaired *t* test.

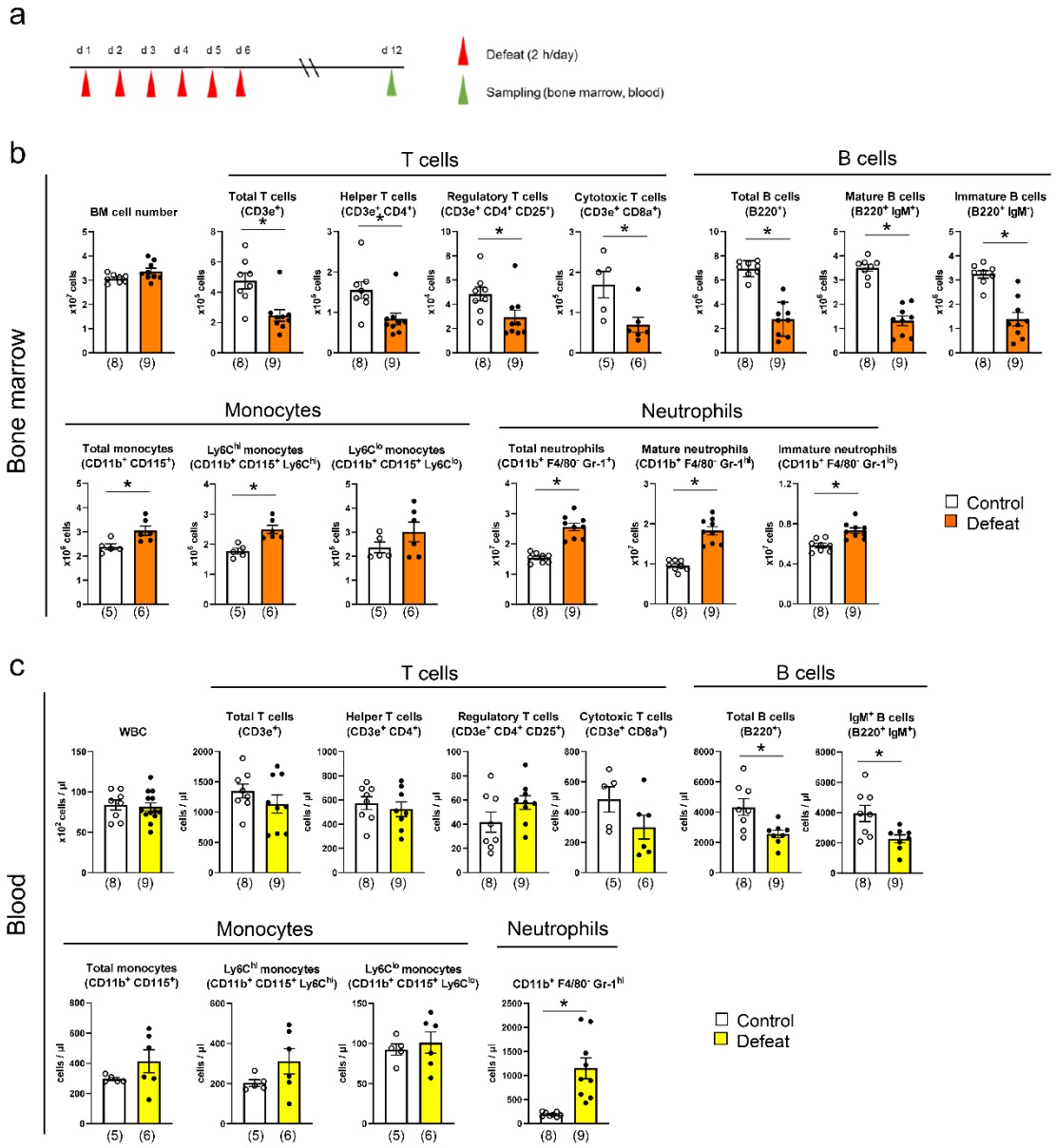


Figure 7. R-SDS-induced neutrophil increase is maintained after the stress in the bone marrow and blood of C57BL/6N mice. (**a**) An experimental schedule. R-SDS with

Protocol 2 is used. A mouse to be defeated is group-housed and exposed to an ICR aggressor mouse for 2 h daily for 6 consecutive days. Cells in the bone marrow and blood were collected at 6 days after the last stress (d12). (**b, c**) The cell numbers of the following leukocyte subsets in the bone marrow (**b**) and blood (**c**) without R-SDS or at 6 days after the last stress (open and orange bars for the bone marrow; open and yellow bars for the blood) are shown: bone marrow (BM) cells, white blood cells (WBC), total T cells ($CD3e^+$), helper T cells ($CD3e^+CD4^+$), regulatory T cells ($CD3e^+CD4^+CD25^+$), cytotoxic T cells ($CD3e^+CD8^+$), total B cells ($B220^+$), mature B cells ($B220^+IgM^+$), immature B cells ($B220^+IgM^-$), total monocytes ($CD11b^+CD115^+$), $Ly6C^{hi}$ monocytes ($CD11b^+CD115^+Ly6C^{hi}$), $Ly6C^{lo}$ monocytes ($CD11b^+CD115^+Ly6C^{lo}$), total neutrophils ($CD11b^+F4/80^-Gr\text{-}1^+$), mature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{hi}$), and immature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{lo}$). Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. $*P <$ 0.05 for unpaired *t* test.

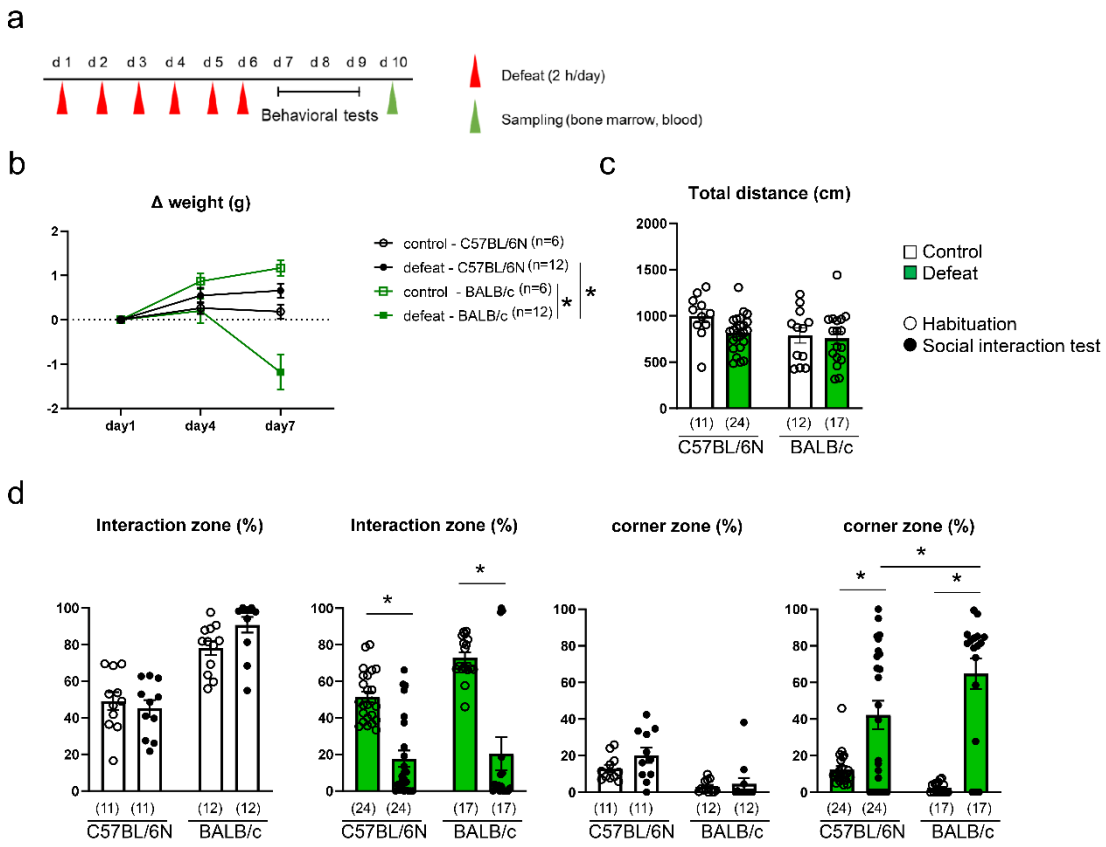


Figure 8. R-SDS-induced social avoidance was larger in BALB/c mice than in C57BL/6N mice. (**a**) An experimental schedule. R-SDS with Protocol 2 is used. A mouse to be defeated is group-housed and exposed to an ICR aggressor mouse for 2 h daily for 6 consecutive days. The social interaction test was performed after R-SDS (d7-9). Cells in the bone marrow and blood were collected at 4 days after the last stress (d10). (**b**) R-SDS-induced reduction in the body weight in BALB/c mice, but not in C57BL/6N mice. Body weights of C57BL/6N (circles) and BALB/c mice (squares) without or with R-SDS (black and green, respectively) were measured at day 1, day 4 and day 7 before each stress exposure. (**c**, **d**) Behavioral results without or with R-SDS (open and green bars, respectively) with Protocol 2. Novelty-induced exploration was measured as the total distance of ambulation during the habituation before the social

interaction test (**c**). The proportions of the times spent in the interaction zone and the corner zone during the social interaction test and its habituation were measured to assess the levels of social interaction and avoidance, respectively (**d**). The number of mice is shown below each bar. Bars represent the mean ± SEM. *$P < 0.05$ for three-way repeated measures ANOVA followed by Tukey's post-hoc test for the comparisons on day 7 (**b**) and for two-way repeated measures ANOVA followed by Bonferroni's post-hoc test for the indicated comparisons (**d**).

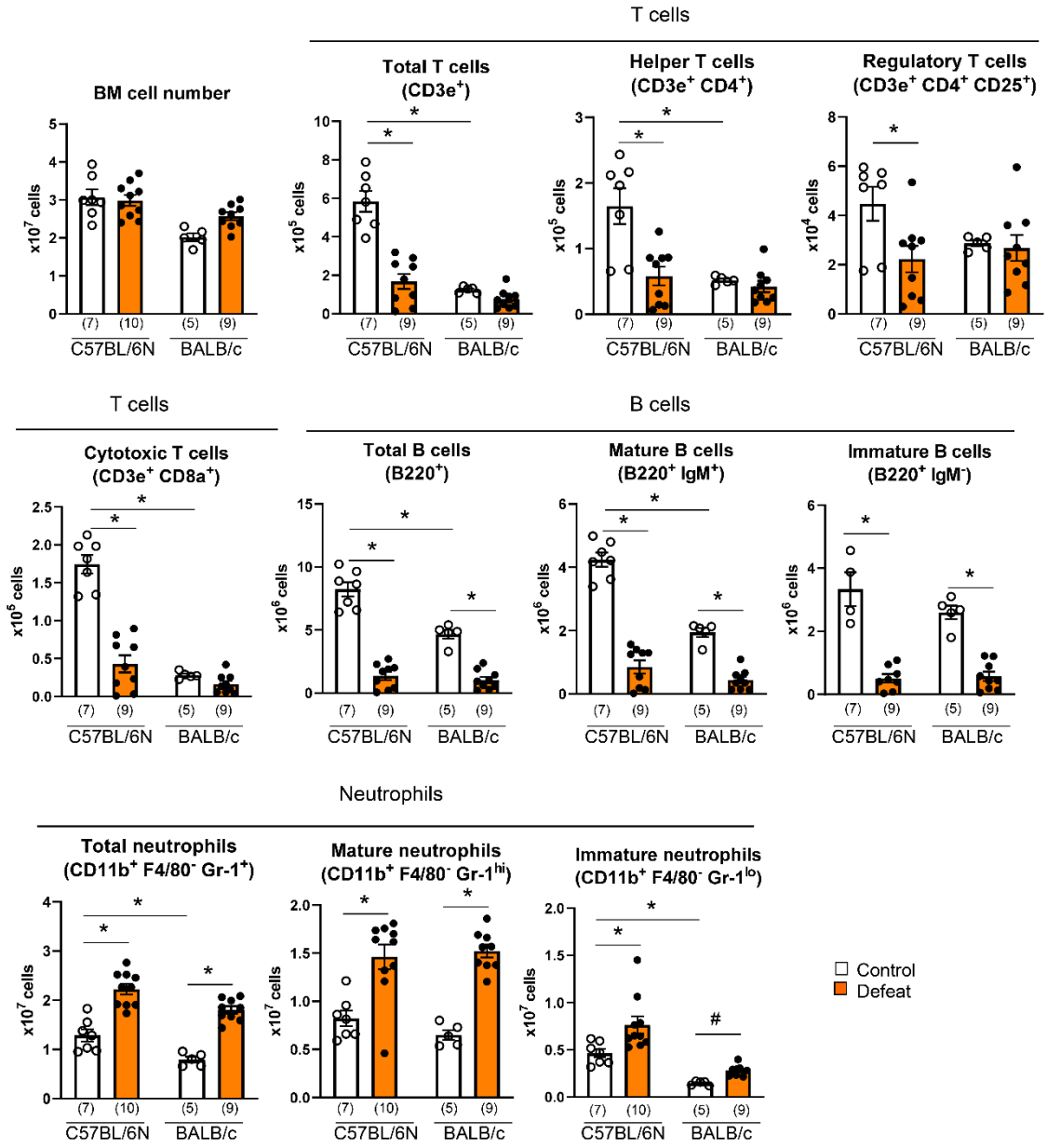


Figure 9. R-SDS increased neutrophils and decreased B cells in the bone marrow of C57BL/6N and BALB/c mice. The effects of R-SDS with Protocol 2 were analyzed. A mouse to be defeated was group-housed and exposed to an ICR aggressor mouse for 2 h daily for 6 consecutive days. Cells in the bone marrow were collected at 4 days after the last stress. The cell numbers of following leukocyte subsets in the bone marrow of each femur without or with R-SDS (open and orange bars, respectively) are shown: bone

marrow (BM) cells, total T cells ($CD3e^+$), helper T cells ($CD3e^+CD4^+$), regulatory T cells ($CD3e^+CD4^+CD25^+$), cytotoxic T cells ($CD3e^+CD8^+$), total B cells ($B220^+$), mature B cells ($B220^+IgM^+$), immature B cells ($B220^+IgM^-$), total neutrophils ($CD11b^+F4/80^-Gr\text{-}1^+$), mature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{hi}$), and immature neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{lo}$). Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. $^*P <$ 0.05 for two-way ANOVA followed by Tukey's post-hoc test. $^{\#}P < 0.05$ for unpaired *t* test.

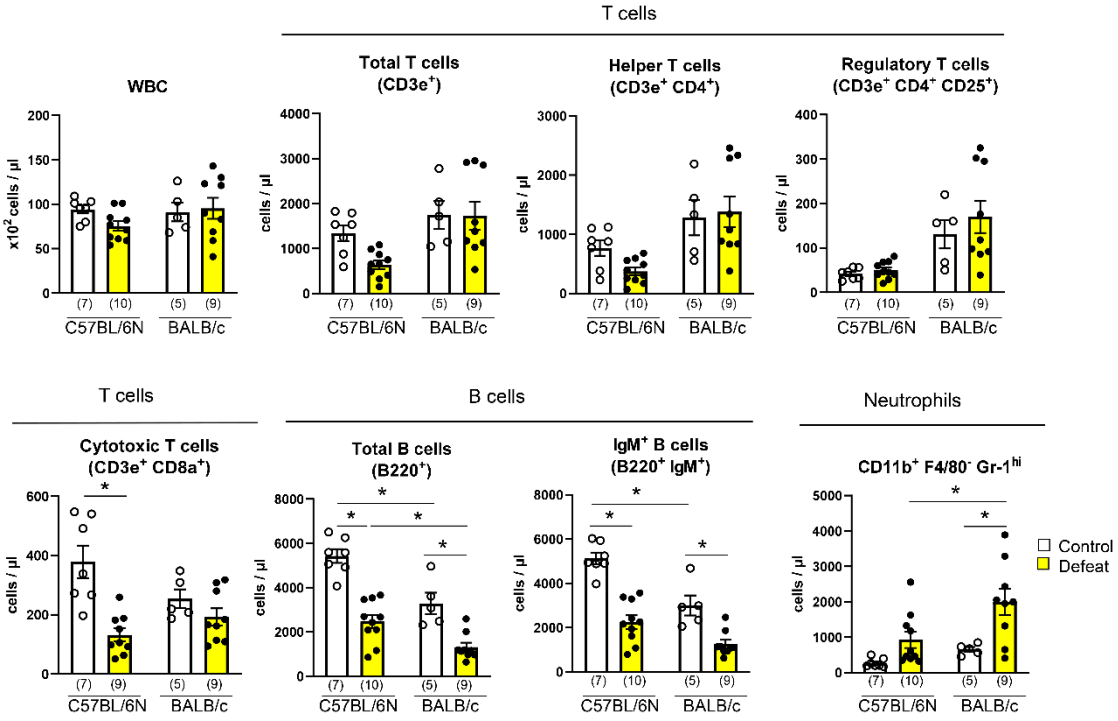
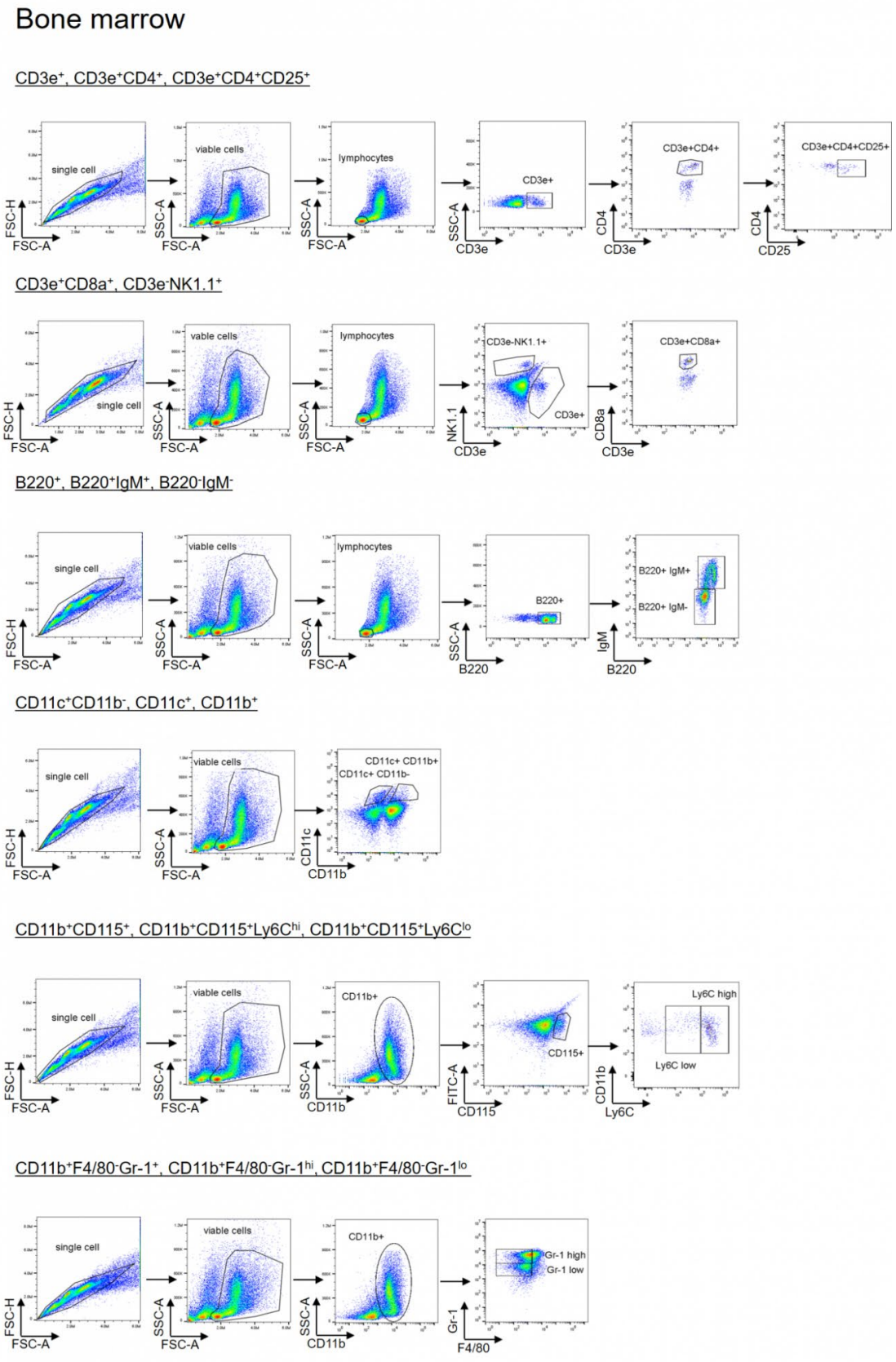
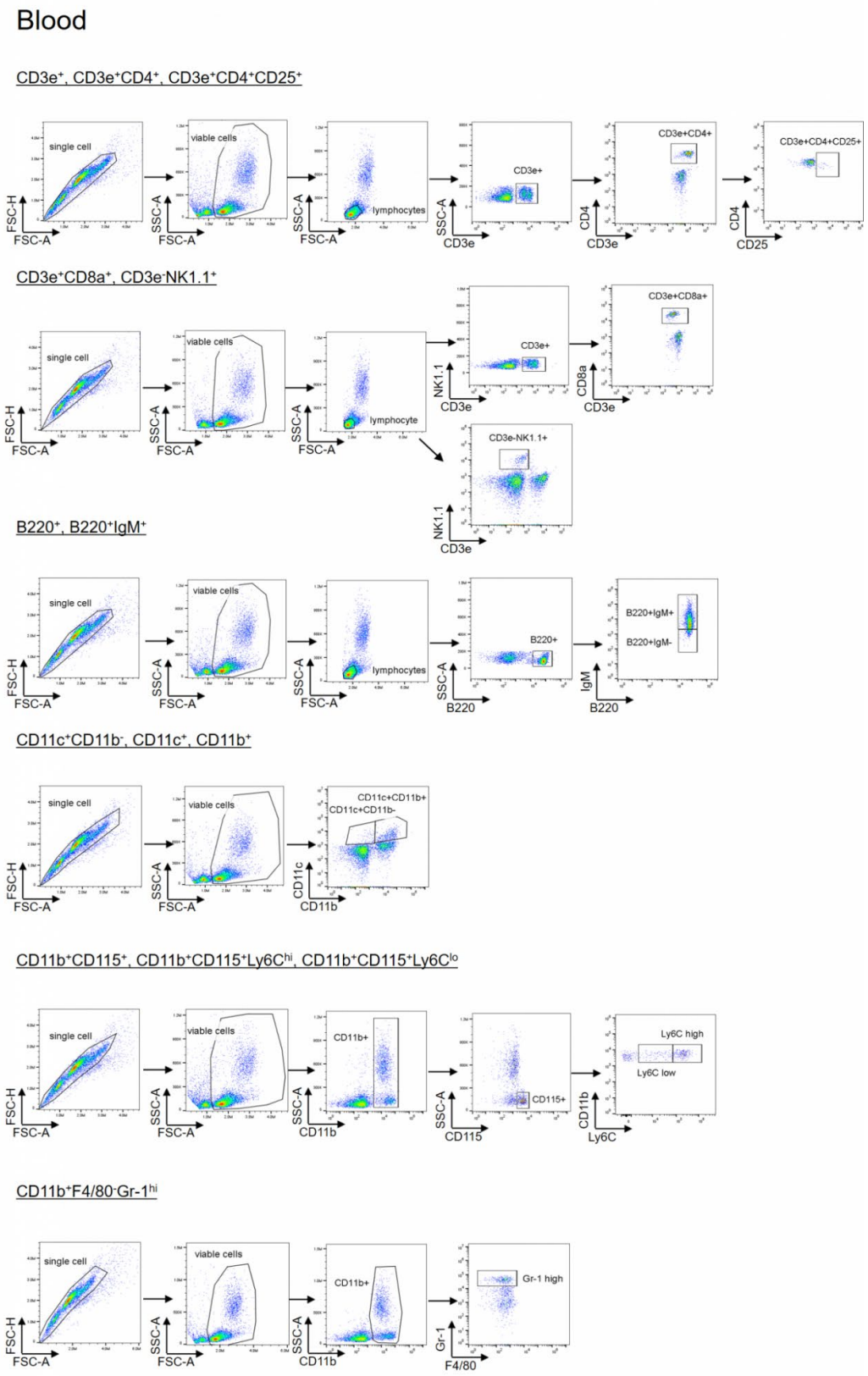


Figure 10. R-SDS-induced increase in circulating neutrophils was larger in BALB/c mice than in C57BL/6N mice. R-SDS with Protocol 2 is used. A mouse to be defeated was group-housed and exposed to an ICR aggressor mouse for 2 h daily

for 6 consecutive days. Cells in the bone marrow and blood were collected at 4 days after the last stress. The cell numbers of following leukocyte subsets in the blood without (open bars) or with (yellow bars) R-SDS are shown: white blood cells (WBC), total T cells ($CD3e^+$), helper T cells ($CD3e^+CD4^+$), regulatory T cells ($CD3e^+CD4^+CD25^+$), cytotoxic T cells ($CD3e^+CD8^+$), total B cells ($B220^+$), IgM-positive B cells ($B220^+IgM^+$), and neutrophils ($CD11b^+F4/80^-Gr\text{-}1^{hi}$). Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. *$P < 0.05$ for two-way ANOVA followed by Tukey's post-hoc test.



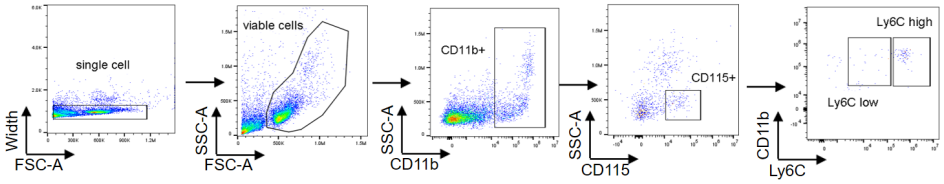
Supplemental Figure 1. The gating strategy for the analyses in Figures 2 and 7b.



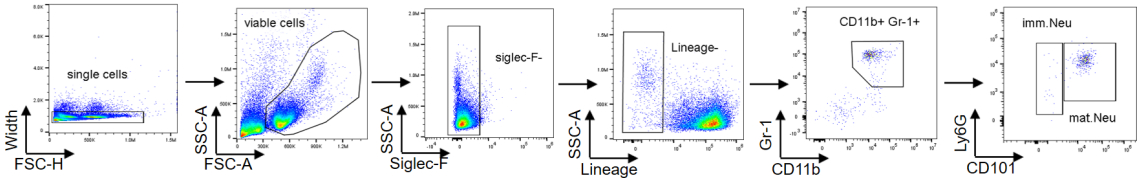
Supplemental Figure 2. The gating strategy for the analyses in Figures 3 and 7c.

Spleen

CD11b⁺CD115⁺, CD11b⁺CD115⁺Ly6C^{hi}, CD11b⁺CD115⁺Ly6C^{lo}



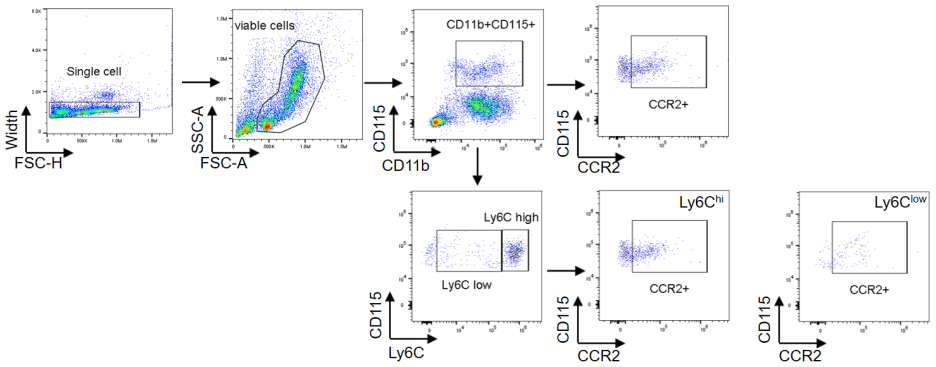
mature neutrophils, immature neutrophils



Supplemental Figure 3. The gating strategy for the analyses in Figure 4.

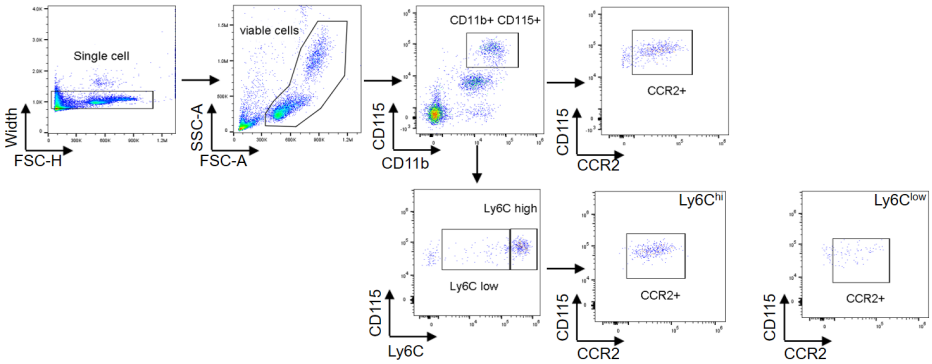
Bone marrow

CD11b^{+}CD115^{+}CCR2^{+}, CD11b^{+}CD115^{+}Ly6C^{hi}CCR2^{+}, CD11b^{+}CD115^{+}Ly6C^{low}CCR2^{+}



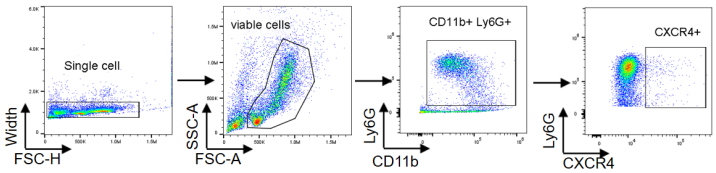
Blood

CD11b^{+}CD115^{+}CCR2^{+}, CD11b^{+}CD115^{+}Ly6C^{hi}CCR2^{+}, CD11b^{+}CD115^{+}Ly6C^{low}CCR2^{+}

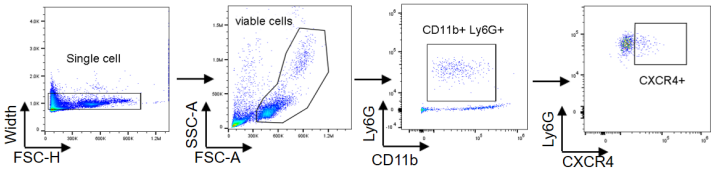


Supplemental Figure 4. The gating strategy for the analyses in Figure 5.

Bone marrow



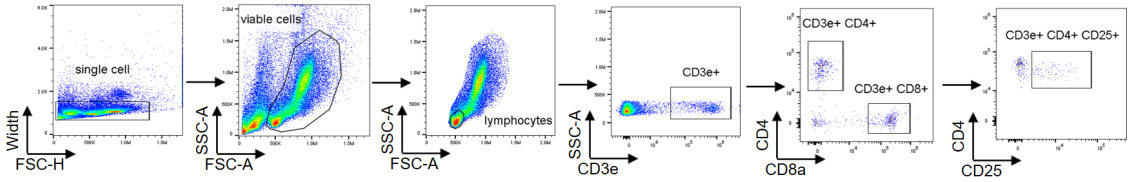
Blood



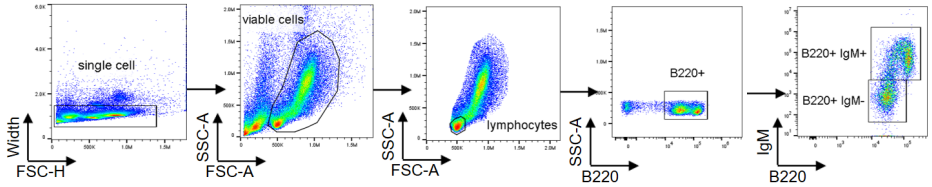
Supplemental Figure 5. The gating strategy for the analyses in Figure 6.

Bone marrow

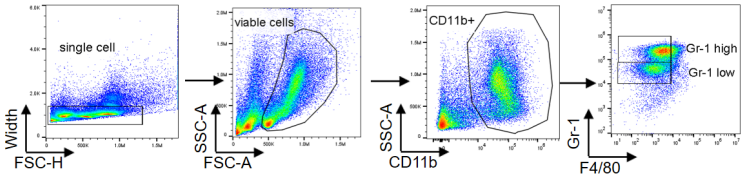
CD3e$^+$, CD3e$^+$CD4$^+$, CD3e$^+$CD4$^+$CD25$^+$, CD3e$^+$CD8a$^+$



B220$^+$, B220$^+$IgM$^+$, B220$^-$IgM$^-$



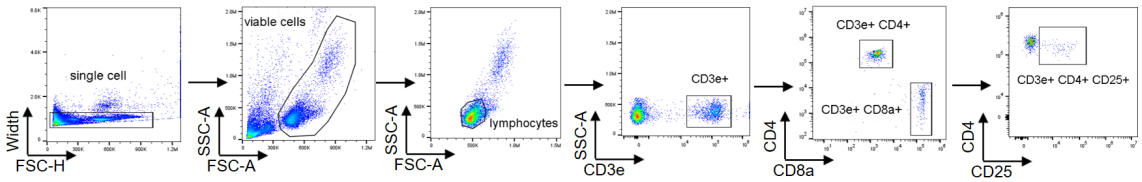
CD11b$^+$F4/80$^-$Gr-1$^+$, CD11b$^+$F4/80$^-$Gr-1^{hi} , CD11b$^+$F4/80$^-$Gr-1^{lo}



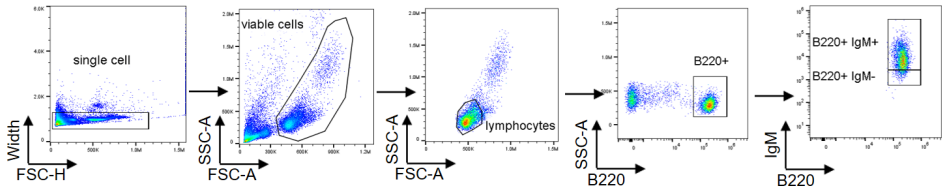
Supplemental Figure 6. The gating strategy for the analyses in Figure 9 and Supplemental Figure 12b.

Blood

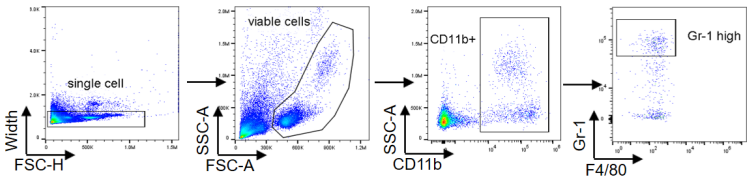
CD3e$^+$, CD3e$^+$CD4$^+$, CD3e$^+$CD4$^+$CD25$^+$, CD3e$^+$CD8a$^+$



B220$^+$, B220$^+$IgM$^+$



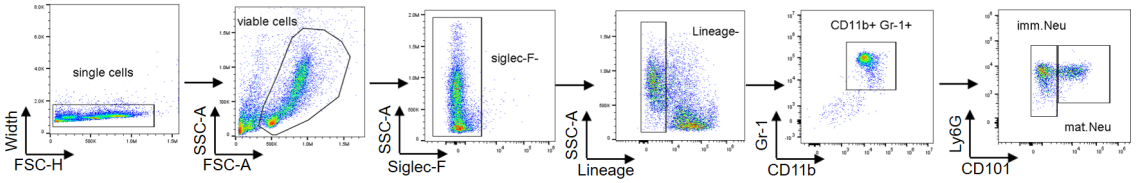
CD11b$^+$F4/80$^-$Gr-1^{hi}



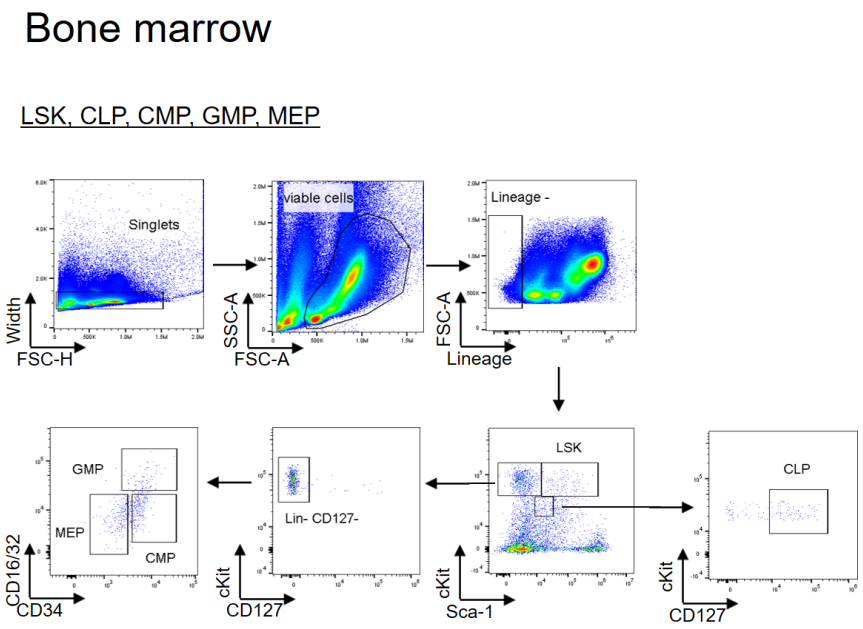
Supplemental Figure 7. The gating strategy for the analyses in Figure 10 and Supplemental Figure 12c.

Bone marrow

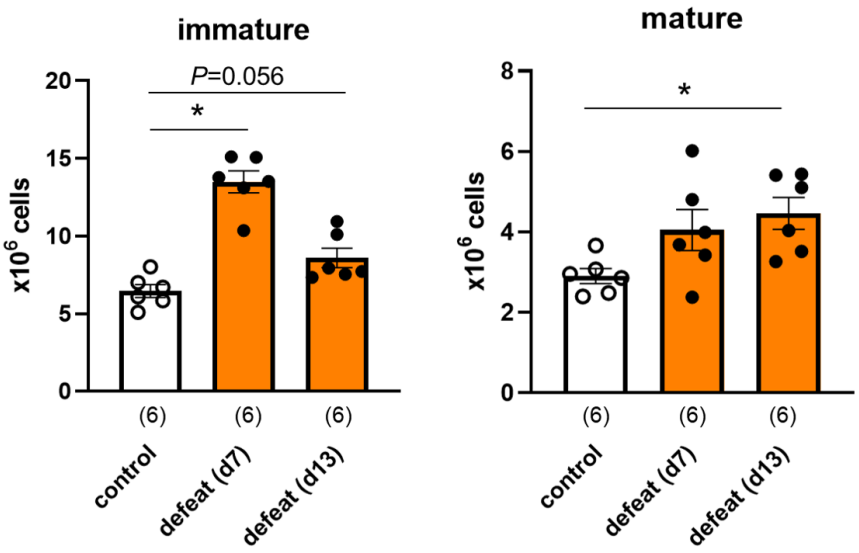
mature neutrophils, immature neutrophils



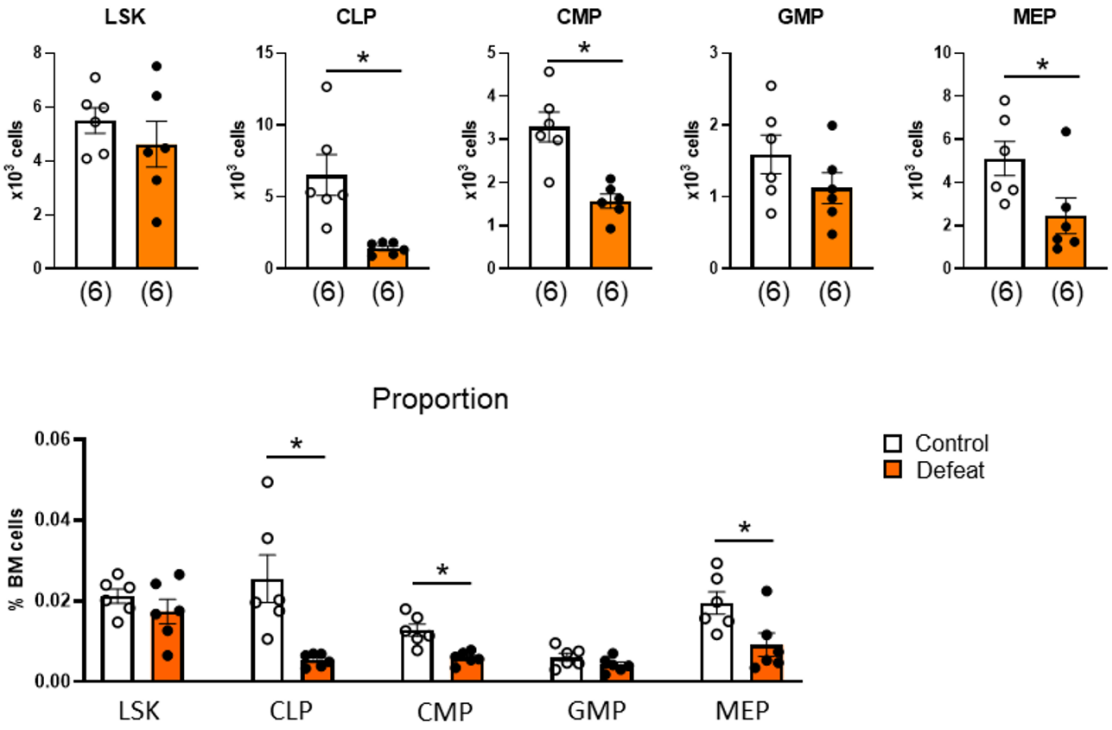
Supplemental Figure 8. The gating strategy for the analyses in Supplemental Figure 10.



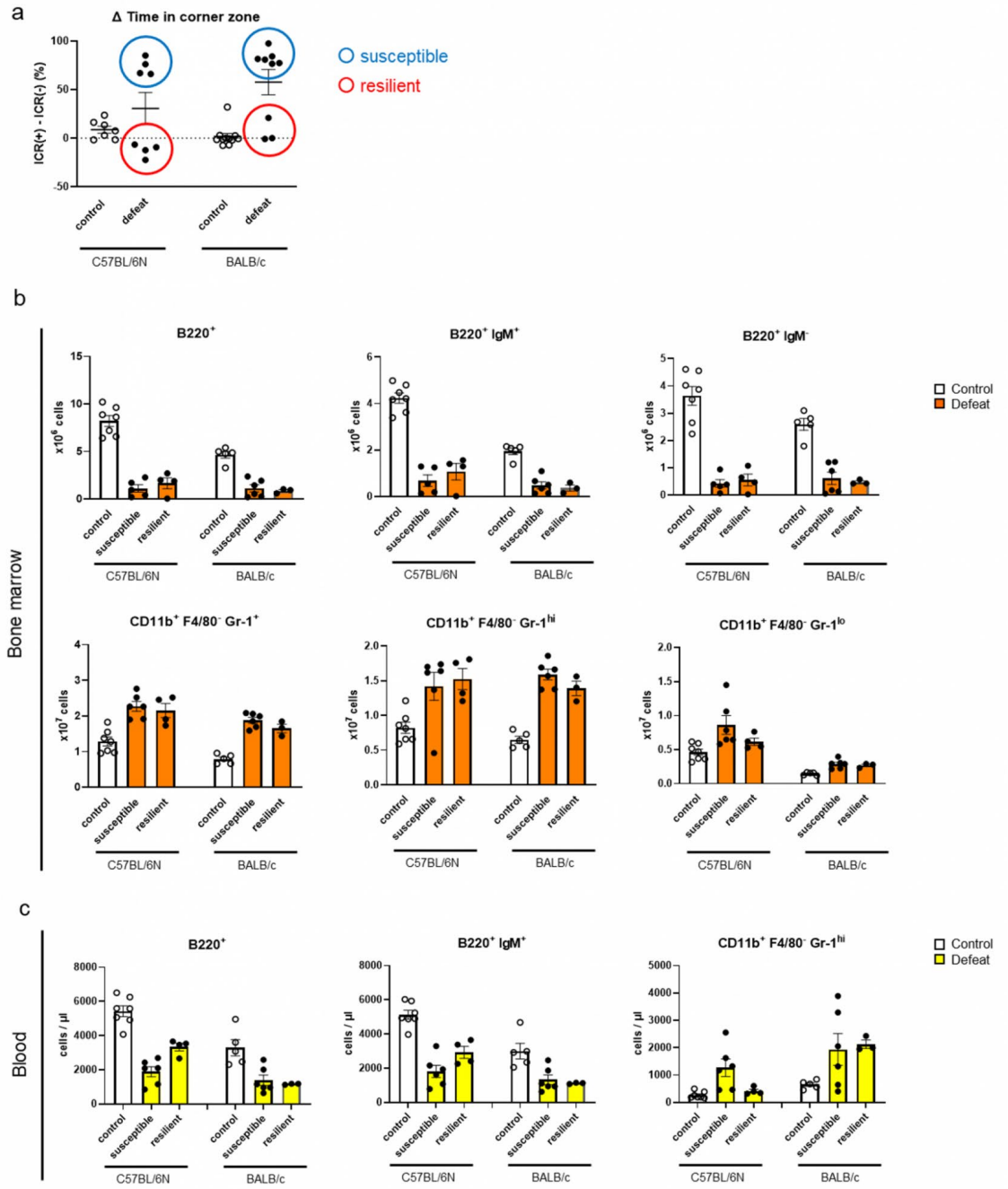
Supplemental Figure 9. The gating strategy for the analyses in Supplemental Figure 11.



Supplemental Figure 10. R-SDS-induced increase in immature and mature neutrophils in the bone marrow was confirmed using a different set of neutrophil markers. The effects of R-SDS with Protocol 2 were analyzed. A mouse to be defeated was group-housed and exposed to an ICR aggressor mouse for 2 h daily for 6 consecutive days. Cells were collected at 12-14 h and 7 days after the last stress (d7 and d13, respectively). The number of immature neutrophils ($Lin^-cKit^-CXCR4^-CD11b^+Gr\text{-}1^+CD101^-$) and mature neutrophils ($Lin^-cKit^-CXCR4^-CD11b^+Gr\text{-}1^+CD101^+$) in the bone marrow were shown. Lineage markers (Lin) used for gating neutrophils are B220, NK1.1, CD90.2, CD115, Siglec-F, and MHCII. Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. *$P < 0.05$ for one-way ANOVA followed by Tukey's post-hoc test.



Supplemental Figure 11. R-SDS decreased myeloid and lymphoid progenitors in the bone marrow of C57BL/6N mice. The effects of R-SDS with Protocol 2 were analyzed. A mouse to be defeated was group-housed and exposed to an ICR aggressor mouse for 2 h daily for 6 consecutive days. Cells in the bone marrow were collected at 12-14 h after the last stress. The cell numbers (**a**) and proportions (**b**) of the following subsets without (open bars) or with (orange bars) R-SDS are shown: LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$), common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte/monocyte progenitor (GMP), and megakaryocyte/erythrocyte progenitor (MEP). Lineage markers (Lin) used for gating myeloid and lymphoid progenitors are B220, Gr-1, CD11b, TER119, CD4, and CD8a. Each dot represents the data point from each mouse. The number of mice is shown below each bar. Bars represent the mean ± SEM. *$P < 0.05$ for unpaired *t* test.



Supplemental Figure 12. No apparent difference between susceptible and resilient mice of C57BL/6N and BALB/c strains was found in R-SDS-induced changes in neutrophils and B cells. The data shown in Figures 8-10 were reanalyzed in relation to the difference between susceptible and resilient mice. The difference between the times spent in the corner zone during the social interaction test (ICR(+)) and those during the habituation (ICR(-)) was used to categorize susceptible and resilient mice (**a**). Only the results of mice used in this figure are shown. Note that the values of susceptible and resilient mice are segregated. Susceptible and resilient mice show similar effects of R-SDS on the number of B cells and neutrophils in the bone marrow (**b**) and blood (**c**). Bars represent the mean ± SEM.

Supplemental Table 1. The proportions of leukocyte subsets shown in Figure 2. Values represent the mean ± SEM in percentage.

	Protocol 1		Protocol 2	
	Control	Defeat	Control	Defeat
$CD3e^+$	4.05 ± 1.26	2.33 ± 0.5	2.69 ± 0.72	1.39 ± 0.48
$CD3e^+CD4^+$	0.94 ± 0.41	0.2 ± 0.08	0.78 ± 0.28	0.32 ± 0.12
$CD3e^+CD4^+CD25^+$	0.28 ± 0.12	0.09 ± 0.04	0.23 ± 0.08	0.1 ± 0.04
$CD3e^+CD8^+$	1.22 ± 0.55	0.29 ± 0.12	1.12 ± 0.37	0.57 ± 0.35
$B220^+$	16.47 ± 2.93	3.81 ± 2.32	23.14 ± 2.57	9.94 ± 4.13
$B220^+IgM^+$	8.71 ± 0.79	2.32 ± 1.44	11.96 ± 0.65	6.01 ± 3.06
$B220^+IgM^-$	7.44 ± 2.33	1.51 ± 0.92	10.71 ± 2.1	3.73 ± 1.14
$NK1.1^+CD3e^-$	0.76 ± 0.09	0.47 ± 0.1	0.81 ± 0.06	0.5 ± 0.15
$CD11c^+CD11b^+$	1.7 ± 0.19	1.03 ± 0.17	1.4 ± 0.34	0.94 ± 0.27
$CD11c^+CD11b^-$	1.9 ± 0.15	1.37 ± 0.38	2.03 ± 0.13	1.61 ± 0.24
$CD11b^+CD115^+$	3.36 ± 1.53	5.32 ± 1.69	3.75 ± 0.92	8.81 ± 1.6
$CD11b^+CD115^+Ly6C^{hi}$	2.77 ± 1.4	4.64 ± 1.64	2.66 ± 0.53	7.24 ± 1.54
$CD11b^+CD115^+Ly6C^{lo}$	0.31 ± 0.15	0.4 ± 0.19	0.47 ± 0.14	0.8 ± 0.12
$CD11b^+F4/80^-Gr\text{-}1^+$	34.45 ± 4.31	58.23 ± 4.28	34.37 ± 3.71	54.12 ± 6.02
$CD11b^+F4/80^-Gr\text{-}1^{hi}$	21.71 ± 3.76	39.47 ± 7.77	20.22 ± 3.12	24.08 ± 6.07
$CD11b^+F4/80^-Gr\text{-}1^{lo}$	12.74 ± 0.87	18.75 ± 7.75	14.14 ± 1.08	30.04 ± 8.65

Supplemental Table 2. The proportions of leukocyte subsets shown in Figure 3. Values represent the mean ± SEM in percentage.

	Protocol 1		Protocol 2	
	Control	Defeat	Control	Defeat
$CD3e^+$	9.93 ± 2.62	6.74 ± 1.37	18.1 ± 2.54	6.5 ± 1.35
$CD3e^+CD4^+$	4.52 ± 0.87	3.33 ± 0.58	9.48 ± 1.26	3.19 ± 0.84
$CD3e^+CD4^+CD25^+$	0.16 ± 0.02	0.13 ± 0.05	0.3 ± 0.07	0.1 ± 0.04
$CD3e^+CD8^+$	5.22 ± 1.77	3.54 ± 1.02	7.14 ± 1.34	2.66 ± 0.39
$B220^+$	29.67 ± 6.11	23.79 ± 6.59	43.03 ± 3.98	5.29 ± 1.68
$B220^+IgM^+$	22.39 ± 8.8	22.74 ± 6.11	38.28 ± 3.79	5.06 ± 1.57
$NK1.1^+CD3e^-$	3.96 ± 0.72	3.17 ± 0.74	3.77 ± 0.78	2.25 ± 1
$CD11c^+CD11b^+$	4.84 ± 1.12	5.33 ± 1.1	4.54 ± 0.64	2.87 ± 0.41
$CD11c^+CD11b^-$	0.63 ± 0.11	0.92 ± 0.52	0.7 ± 0.19	0.29 ± 0.07
$CD11b^+CD115^+$	6.26 ± 1.58	8.56 ± 2.54	6.52 ± 1.15	12.82 ± 2.82
$CD11b^+CD115^+Ly6C^{hi}$	3.21 ± 1.08	4.93 ± 1.77	2.87 ± 0.51	10.77 ± 2.44
$CD11b^+CD115^+Ly6C^{lo}$	1.05 ± 0.28	0.84 ± 0.33	1.66 ± 0.61	0.44 ± 0.06
$CD11b^+F4/80^-Gr\text{-}1^{hi}$	8.49 ± 2.5	16.95 ± 4.93	3.92 ± 1.39	37.35 ± 5.46

Supplemental Table 3. The proportions of leukocyte subsets shown in Figure 4. Values represent the mean ± SEM in percentage.

	Control	Defeat (day 7)
$CD11b^{+}CD115^{+}$	1.58 ± 0.31	2.72 ± 1.26
$CD11b^{+}CD115^{+}Ly6C^{hi}$	0.65 ± 0.15	1.64 ± 0.77
$CD11b^{+}CD115^{+}Ly6C^{lo}$	0.9 ± 0.19	1.07 ± 0.5
$Lin^{-}cKit^{-}CXCR4^{-}CD11b^{+}Gr\text{-}1^{+}$	2.09 ± 0.44	8.87 ± 4.19
immature neutrophils	0.21 ± 0.04	2.04 ± 1.72
mature neturophils	1.87 ± 0.43	6.75 ± 3.61

Supplemental Table 4. The proportions of leukocyte subsets shown in Figure 7. Values represent the mean ± SEM in percentage.

	Bone marrow		Blood	
	Control	Defeat	Control	Defeat
$CD3e^+$	0.79 ± 0.21	0.35 ± 0.1	15.4 ± 2.66	13.91 ± 3.69
$CD3e^+CD4^+$	0.29 ± 0.08	0.12 ± 0.02	6.8 ± 1.11	6.34 ± 1.48
$CD3e^+CD4^+CD25^+$	0.11 ± 0.03	0.08 ± 0.05	0.44 ± 0.2	0.66 ± 0.16
$CD3e^+CD8^+$	0.59 ± 0.22	0.23 ± 0.12	5.58 ± 2.22	4.01 ± 1.96
$B220^+$	17.43 ± 2.74	7.86 ± 3.06	50.76 ± 9.29	29.93 ± 14.11
$B220^+IgM^+$	9.49 ± 1.38	6.86 ± 2.95	46.39 ± 10.68	26.78 ± 14.21
$B220^+IgM^-$	7.78 ± 1.41	0.08 ± 0.04	-	-
$CD11b^+CD115^+$	8.27 ± 0.54	10.21 ± 1.06	3.38 ± 0.68	5.53 ± 1.9
$CD11b^+CD115^+Ly6C^{hi}$	6.13 ± 0.49	8.37 ± 0.87	2.33 ± 0.72	4.13 ± 1.63
$CD11b^+CD115^+Ly6C^{lo}$	0.82 ± 0.12	0.99 ± 0.24	1.02 ± 0.09	1.37 ± 0.32
$CD11b^+F4/80^-Gr\text{-}1^+$	50.97 ± 2.83	80.98 ± 6.6	-	-
$CD11b^+F4/80^-Gr\text{-}1^{hi}$	32.15 ± 2.81	58.55 ± 5.44	2.29 ± 0.77	13.58 ± 5.98
$CD11b^+F4/80^-Gr\text{-}1^{lo}$	18.82 ± 1.26	22.43 ± 2.08	-	-

Supplemental Table 5. The proportions of leukocyte subsets shown in Figure 9. Values represent the mean ± SEM in percentage.

	C57BL/6N		BALB/c	
	Control	Defeat	Control	Defeat
$CD3e^+$	1.89 ± 0.22	0.6 ± 0.41	0.62 ± 0.06	0.29 ± 0.16
$CD3e^+CD4^+$	0.45 ± 0.24	0.21 ± 0.15	0.26 ± 0.04	0.16 ± 0.09
$CD3e^+CD4^+CD25^+$	0.13 ± 0.05	0.08 ± 0.06	0.14 ± 0.02	0.1 ± 0.05
$CD3e^+CD8^+$	0.58 ± 0.12	0.15 ± 0.12	0.14 ± 0.01	0.06 ± 0.05
$B220^+$	27.21 ± 5.01	4.82 ± 3.28	23.04 ± 2.13	3.95 ± 2.48
$B220^+IgM^+$	13.96 ± 1.51	3.01 ± 2.1	9.61 ± 0.94	1.67 ± 1.04
$B220^+IgM^-$	12.33 ± 3.29	1.69 ± 1.23	12.79 ± 1.26	2.17 ± 1.48
$CD11b^+F4/80^-Gr\text{-}1^+$	41.59 ± 4.37	74.68 ± 5.03	39.3 ± 2.66	70.28 ± 4.73
$CD11b^+F4/80^-Gr\text{-}1^{hi}$	26.59 ± 3.09	48.48 ± 10.51	32.06 ± 2.27	59.35 ± 5.04
$CD11b^+F4/80^-Gr\text{-}1^{lo}$	15 ± 1.63	26.2 ± 11.59	7.25 ± 0.75	10.93 ± 1.5

Supplemental Table 6. The proportions of leukocyte subsets shown in Figure 10. Values represent the mean ± SEM in percentage.

	C57BL/6N		BALB/c	
	Control	Defeat	Control	Defeat
$CD3e^+$	13.98 ± 3.55	8.58 ± 3.74	20.31 ± 8.18	17.33 ± 3.93
$CD3e^+CD4^+$	7.96 ± 2.88	5.02 ± 2.48	15.15 ± 7.5	13.71 ± 3.53
$CD3e^+CD4^+CD25^+$	0.44 ± 0.1	0.67 ± 0.23	1.54 ± 0.81	1.64 ± 0.54
$CD3e^+CD8^+$	3.95 ± 1.09	2.04 ± 1.1	2.79 ± 0.29	2.04 ± 0.44
$B220^+$	57.7 ± 5.48	34.23 ± 13.87	35.69 ± 2.61	15.08 ± 7
$B220^+IgM^+$	54.72 ± 6.19	31.22 ± 13.18	32.61 ± 3.57	14.44 ± 6.52
$CD11b^+F4/80^-Gr\text{-}1^{hi}$	2.87 ± 1.13	11.89 ± 7.97	7.27 ± 0.96	19.78 ± 6.66