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Full paper

Chronic stress alters lipid mediator profiles associated with immune-related gene expressions and cell compositions in mouse bone marrow and spleen

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ABSTRACT

Despite the importance of lipid mediators in stress and depression and their link to inflammation, the influence of stress on these mediators and their role in inflammation is not fully understood. This study used RNA-seq, LC-MS/MS, and flow cytometry analyses in a mouse model subjected to chronic social defeat stress to explore the effects of acute and chronic stress on lipid mediators, gene expression, and cell population in the bone marrow and spleen. In the bone marrow, chronic stress induced a sustained transition from lymphoid to myeloid cells, accompanied by corresponding changes in gene expression. This change was associated with decreased levels of 15-deoxy-d12,14-prostaglandin J_2, a lipid mediator that inhibits inflammation. In the spleen, chronic stress also induced a lymphoid-to-myeloid transition, albeit transiently, alongside gene expression changes indicative of extramedullary hematopoiesis. These changes were linked to lower levels of 12-HEPE and resolvins, both critical for inhibiting and resolving inflammation. Our findings highlight the significant role of anti-inflammatory and pro-resolving lipid mediators in the immune responses induced by chronic stress in the bone marrow and spleen. This study paves the way for understanding how these lipid mediators contribute to the immune mechanisms of stress and depression.

1. Introduction

Stress, a response to challenging conditions, impacts mental and physical functions in various ways based on its nature. Acute stress, at optimal levels, can elicit adaptive responses, bolstering resilience and well-being, whereas chronic or severe stress can disturb mental and physical functions, increasing the risk of various illnesses, including depression.[1] Rodent models have been instrumental in elucidating these biological mechanisms, highlighting the distinct outcomes of acute and chronic stress.[2–7]

Clinical research links inflammation with depressive disorders, as evidenced by elevated proinflammatory cytokines in patients and brain inflammation observed via PET imaging.[8,9] Chronic stress, especially as modeled by chronic social defeat stress in mice, incites inflammation both in and outside the brain, leading to depression-related behaviors. This effect is mediated by microglial activation via innate immune receptors TLR2/4, possibly triggered by neuronally derived ligands like HMGB1.[10,11] Concurrently, chronic stress activates the sympathetic nervous and neuroendocrine systems, mobilizing neutrophils and monocytes from the bone marrow to the bloodstream.[12–16] Recent evidence highlights crucial roles of myeloid cell mobilization for chronic stress-induced behavioral changes. For example, genetic deletions in

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chemokine receptors CCR2 and CX3CR1 reduce both monocyte infiltration into the brain and anxiety-like behavior due to chronic stress.[14] The transplantation of bone marrow cells lacking caspase-1, a protein crucial for IL-1β production, diminishes monocyte infiltration into the brain and anxiety-like behaviors.[15] In addition, chronic stress causes hematopoietic stem/progenitor cells to migrate from the bone marrow to the spleen for extramedullary hematopoiesis.[16] Extramedullary hematopoiesis in the spleen is thought to be crucial for stress sensitization, an exacerbation of behavioral responses to stress with prior stress exposure since it is abolished by splenectomy.[17,18] Chronic stress also compromises the blood-brain barrier by reducing the expression of the endothelial tight junction protein claudin 5, thereby allowing peripheral blood cells and cytokines to influence the brain.[19,20] Given that IL-1 receptor type 1 knockdown in vascular endothelial cells reportedly attenuate chronic stress-induced anxiety-like behavior, IL-1β derived from myeloid cells may be involved in the blood-brain barrier disruption.[21] These findings indicate that chronic stress induces the mobilization of myeloid cells from the bone marrow and their brain infiltration through the disrupted blood-brain barrier, contributing to behavioral alterations. However, the molecular mechanisms driving this stress-induced inflammation are not yet fully understood.

The role of lipid mediators in inflammation and mental health has gained attention, particularly with the discovery that NSAIDs like celecoxib, which inhibit prostaglandin (PG) synthesis, enhance the efficacy of antidepressants.[22] Rodent studies indicate that chronic stress increases PGE_2 synthesis, leading to the suppression of dopamine neurons, while leukotrienes have been implicated in brain inflammation and depression-related behaviors.[4,23,24] Technological advances in LC-MS/MS have enabled the identification of numerous previously undetectable lipid mediators involved in inflammation.[25,26] These include a variety of anti-inflammatory and pro-resolving lipid mediators, such as lipoxins, resolvins, protectins, and maresins, mostly derived from ω3 PUFAs, with some from ω6 PUFAs.[27] Elevated levels of ω3 PUFAs have been associated with a reduced risk of depression, and their supplementation appears to alleviate depression or its behavioral manifestations in humans and rodents.[28–30] Pro-resolving metabolites from ω3 PUFAs counteract these behaviors, likely through their anti-in flammatory/pro-resolving effects.[31,32] Other anti-inflammatory/pro-resolving lipid mediators, including 15-deoxy-d12,14 PGJ_2 and 12-HEPE, remain unexplored in the context of stress and mental health.[33,34]

Recognizing the significance of inflammation and lipid mediators in stress and depression, our prior studies focused on the transcriptome of microglia and LC-MS/MS analyses of the brain under chronic stress.[35,36] The impact of chronic stress on lipid mediators in peripheral tissues, such as bone marrow and spleen, however, remained unexamined. This study aims to bridge this gap by conducting transcriptome, flow cytometry, and lipidome analyses on these tissues, evaluating alterations across multiple stress conditions. Our findings reveal distinct, coordinated shifts in immune-related gene expressions, cell compositions, and lipid mediator profiles in bone marrow and spleen in response to acute and chronic stress.

2. Materials and methods

2.1. Animals

We obtained adult male C57BL/6 N mice aged 9–10 weeks and male ICR mice, retired from breeding, from Japan SLC (Shizuoka, Japan). These mice were housed in a controlled animal facility with stable temperature and humidity, maintained on a 12-h light/dark cycle, and had unlimited access to food and water. Initially, the mice were grouped in cages, housing 4–5 each. One week before the behavioral experiments commenced, they were housed individually. All animal care and experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and received approval from the Animal Care and Use Committee of Kobe University Graduate School of Medicine (No. P200201).

2.2. Chronic social defeat stress

We conducted chronic social defeat stress experiments with two cohorts of mice, following previously established procedures with minor modifications (Fig. 1a).[3,35] Prior to these experiments, we assessed the aggressiveness of male ICR mice toward a novel male C57BL/6 N mouse by monitoring attack latency and frequency over three 3-min daily sessions across three days. Only sufficiently aggressive ICR mice were selected for inclusion. During the social defeat stress procedure, an experimental C57BL/6 N mouse was introduced into the home cage of an aggressive male ICR mouse. This mouse was then subjected to daily 10-min attack sessions for the specified duration. Following each 10-min exposure to social defeat stress, the stressed mice were either immediately euthanized for tissue sampling (Fig. 1b) or left undisturbed until the next stress exposure or the social interaction test.

In both experimental cohorts, male C57BL/6 N mice were categorized into five groups: Control (N = 4), 1xStress (N = 4), 4xStress (N = 4), 10xStress (N = 6), and 10xStress + Recovery (N = 4). Control mice were not subjected to social defeat stress, left undisturbed in their home cages. The 1xStress, 4xStress, and 10xStress groups experienced one, four, and ten sessions of social defeat stress, respectively, each lasting 10 min daily. These mice were euthanized immediately after their last stress session for tissue sampling. The 10xStress + Recovery group underwent one week of recovery following the tenth stress session before tissue sampling. Both the Control and 10xStress + Recovery groups were euthanized immediately after the social interaction test. Collected tissues were used for RNA-seq-based transcriptome and LC-MS/MS-based lipidome analyses in the first cohort, and for flow cytometry analysis in the second cohort.

2.3. Social interaction test

The social interaction test was conducted according to established protocols.[3,5,35] One day prior to the initial test, each experimental mouse was habituated for 150 s in a 30 cm × 40 cm open rectangular chamber containing an empty metal mesh enclosure at one end. During the social interaction test, the experimental mouse was reintroduced into the same chamber for an additional 150 s. This time, a novel ICR male mouse was enclosed within the metal meshwork. Behavioral observations were video-recorded and later analyzed using SMART video tracking software (PanLab Harvard Apparatus, Holliston, MA, USA). We defined the areas surrounding the ICR mouse and those at the opposite end as the interaction and avoidance zones, respectively (Fig. 1c). The time spent in each zone was quantified to assess stress-induced social avoidance.

2.4. Tissue sampling

The mice were deeply anesthetized with isoflurane, and samples of femur bone marrow and spleen were collected for further analyses. In performing lipidome and transcriptome analysis, one femur from each mouse was used for LC-MS/MS-based lipidome analysis, and the other for RNA-seq-based transcriptome analysis. For the lipidome analysis of femur bone marrow, the femur's proximal end was sectioned, and the bone marrow flushed with −20 °C methanol, then stored at −80 °C until use, as previously described.[37] For the transcriptome analysis, the bone marrow was flushed with ice-cold RPMI buffer, followed by centrifugation at 1500 rpm for 5 min at 4 °C. The resultant pellet was collected, and RNA was purified using the NucleoSpin RNA Plus kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Half of each spleen was preserved in RNAlater solution (Thermo Fisher Scientific), and RNA was purified using the same kit. The other half of the spleen was flash-frozen in liquid nitrogen for lipidome analysis. To perform flow cytometry, one femur bone and the spleen were harvested.

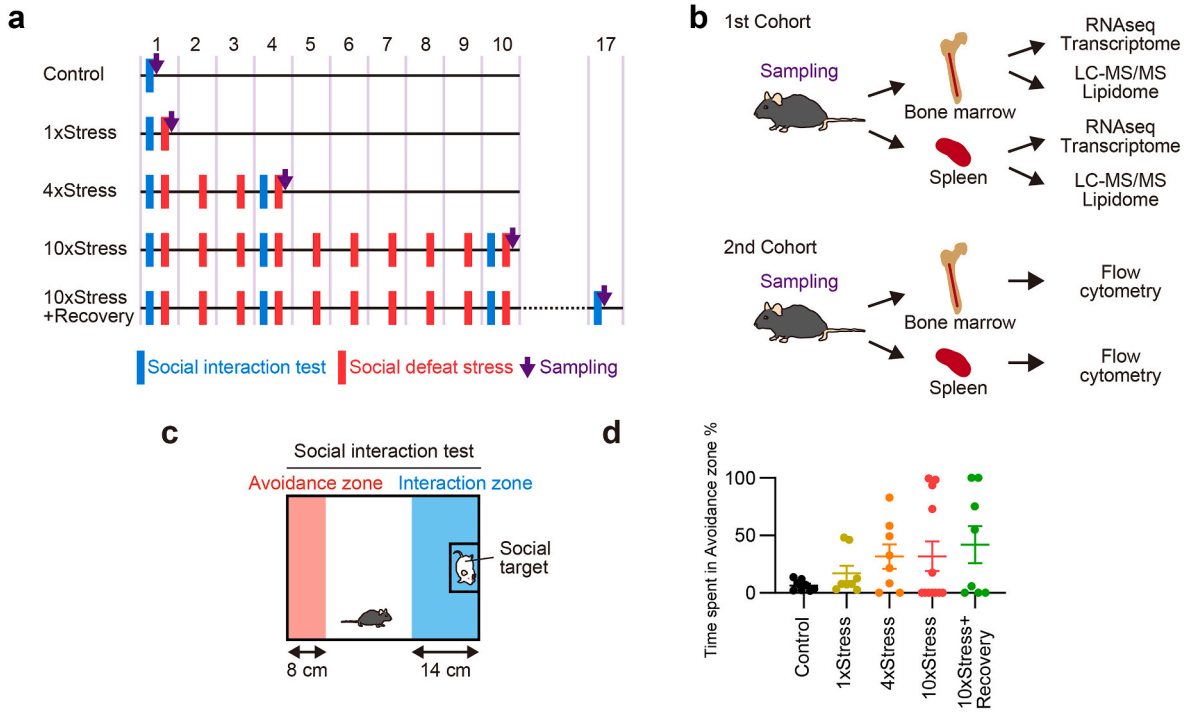

Fig. 1. Collection and Analysis of Bone Marrow and Spleen Samples During Chronic Social Defeat Stress
(a) Experimental timeline. Mice were subjected to 10 min of daily social defeat stress over a period of 10 days. Social interaction tests were conducted on Days 1, 4, 10, and 17. Tissue samples were collected at key time points: immediately following the first social interaction test for Control mice, after the initial exposure to social defeat stress for 1xStress mice, after the fourth exposure for 4xStress mice, after the tenth exposure for 10xStress mice, and after the Day 17 social interaction test for 10xStress + Recovery mice. (b) Tissue collection. Bone marrow and spleen tissues were obtained from each mouse. In the first cohort, half of each tissue sample was utilized for RNA-seq-based transcriptome analysis, while the remaining half was employed for LC-MS/MS-based lipidome analysis. In the second cohort, tissue sample was utilized for flow cytometry analysis. (c) Behavioral testing chamber, utilized for the social interaction test, depicting the social avoidance zone in red and the social interaction zone in blue. (d) Behavioral analysis. Time spent in the avoidance zone during the final social interaction test, presented for each mouse group. Data are represented as means ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The femur was flushed with ice-cold RPMI buffer. The spleen was gently dissociated and filtered through a 70-μm strainer. The resultant cells from each tissue were subjected to flow cytometry.

2.5. Lipidome analysis

Lipidome analysis was conducted following established procedures.[25,35,38] Frozen spleen tissue was homogenized prior to processing. Both spleen and bone marrow samples were treated with an excess of ice-cold methanol and deuterated internal standards (500 pg each), including d4-LTB_4, d8-5-HETE, d4-PGE_2, and d5-RvD_2. The samples were incubated at 4 °C for 1 h, then centrifuged at 15,000 rpm for 5 min at 4 °C. Lipid metabolites were solid-phase extracted on C18 columns and quantified using LC-MS/MS (Qtrap 6500, SCIEX, USA). LC-MS/MS peaks with poor signal-to-noise ratio (S/N < 10) were excluded from quantification. For further analysis, the amount of each metabolite was normalized to the average amount in control mice, which had not been subjected to chronic social defeat stress. The data are available in Supplementary Tables 1 and 2 The conversion ratio of 15-deoxy-d12,14 PGJ2, defined as the amount of this metabolite relative to its precursor arachidonic acid, was calculated to investigate the impact of stress on its synthesis pathway.

2.6. RNA-seq analysis

The quality of purified RNA was initially assessed using the RNA pico chip on the Bioanalyzer 2100 (Agilent, USA). The RNA Integrity Number (RIN) for samples used in RNA-seq analysis averaged 7.30 ± 0.17 (mean ± SEM, N = 35). RIN values were not determined for two samples due to their low RNA concentration. These RNA samples were processed according to the manufacturer's instructions using the Illumina TruSeq Stranded mRNA Library Prep kit and IDT for Illumina – TruSeq RNA UD Indexes v2. After purification, the libraries were evaluated with a High Sensitivity DNA chip on the Bioanalyzer 2100, then pooled. RNA-seq analysis was performed on a Novaseq X Plus platform by Novogene Co., Ltd. (Beijing, China). The samples whose library DNA was not detectable with Bioanalyzer 2100 (i.e., less than 0.7 nM) were excluded from the analysis. This included one bone marrow-derived RNA sample in the Control group, one in the 4xStress group, one in the 10xStress group, two spleen-derived RNA samples in the 1xStress group, and two in the 4xStress group. Consequently, the sample sizes for transcriptome analysis were: in the bone marrow, N = 3 for Control, N = 4 for 1xStress, N = 3 for 4xStress, N = 5 for 10xStress, and N = 4 for 10xStress + Recovery; in the spleen, N = 4 for Control, N = 2 for 1xStress, N = 2 for 4xStress, N = 6 for 10xStress, and N = 4 for 10xStress + Recovery. Read quantification was performed using Kallisto (version 0.46.1) with mouse cDNA sequences from Ensemble GRCm38. Differential gene expression analysis was conducted using Sleuth, with quantified transcript expression levels in TPM (transcripts per million) merged to determine the expression of a given gene. The data were deposited to Gene Expression Omnibus (GEO) database under accession number GSE254978.

Genes detectable in all Control mice were selected for further analysis. The Z-score of each gene's expression was computed, and cluster analysis was performed using the hierarchical clustering algorithm. For the Gene Ontology analysis in Fig. 2b and c,3b-d, we analyzed genes showing statistically significant differential expression ($P < 0.05$) and a fold change >2 or <0.5 when comparing the Control and 10xStress groups. The Gene Ontology analysis utilized the publicly available resource at https://geneontology.org/. The Gene Ontology terms that

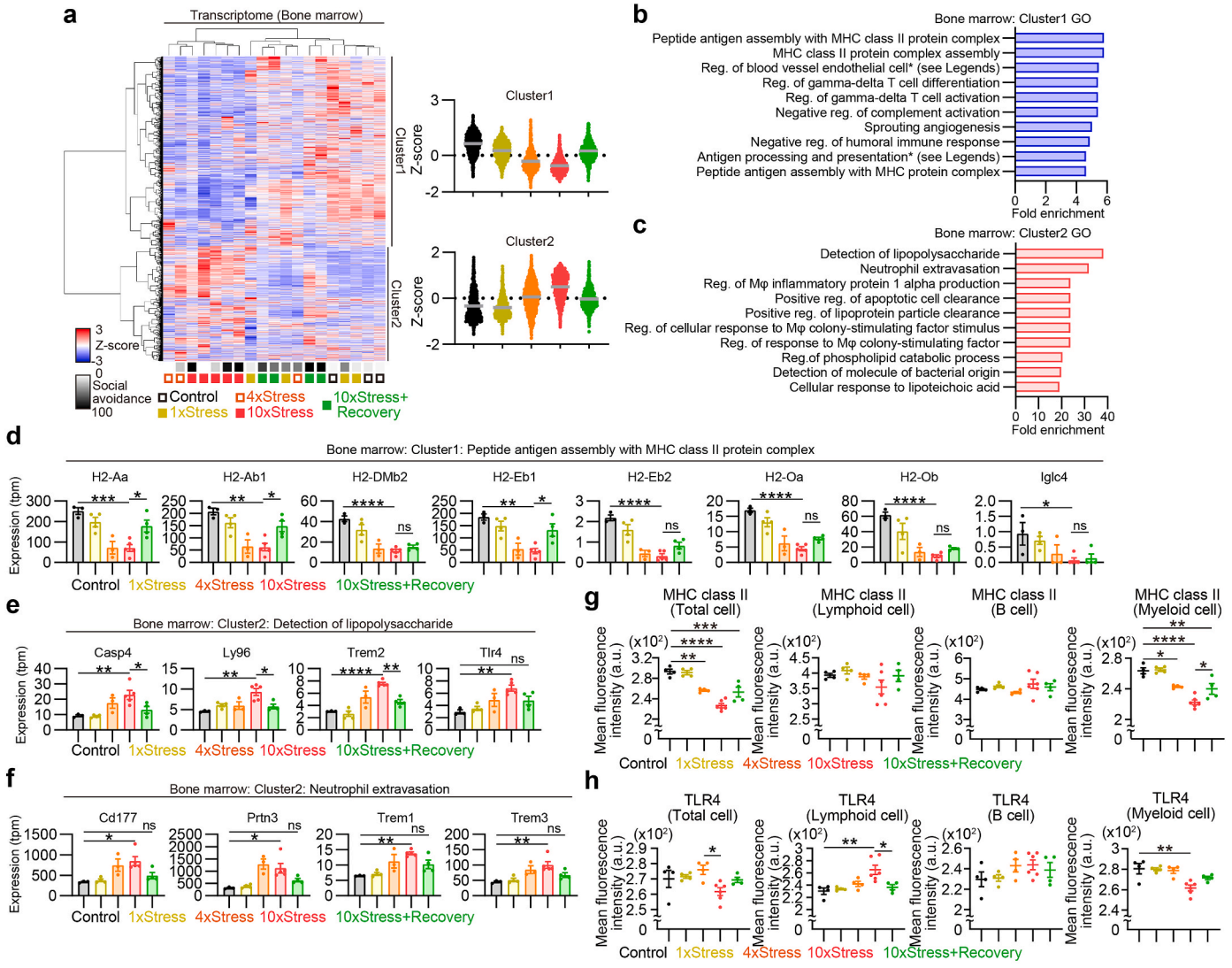

Fig. 2. Dysregulation of Immune-Related Gene Expression in the Bone Marrow due to Chronic Stress

(a) Cluster analysis in the bone marrow transcriptome. The heat map (left) and bar graphs (right) illustrate Z-scores for genes in Clusters 1 and 2. Below, mouse groups and their social avoidance levels are presented. Genes in Cluster 1 show downregulation during chronic stress, whereas those in Cluster 2 show upregulation. (b,c) Gene Ontology terms for Clusters 1 and 2. These include terms linked to genes in Cluster 1 (b) and Cluster 2 (c), associated with statistically significant differences and a fold change of >2 or <0.5 when comparing the 10xStress and Control groups. "Reg." represent Regulation. In panel b, the third and ninth entries are "Regulation of blood vessel endothelial cell proliferation involved in sprouting angiogenesis" and "antigen processing and presentation of exogenous peptide antigen via MHC class II," respectively. (d–f) Gene expression levels in selected Gene Ontology terms. This shows the expression levels of genes within the terms "Peptide antigen assembly with MHC class II protein complex" (d), "Detection of lipopolysaccharide" (e), and "Neutrophil extravasation" (f) from the lists in (b,c). (g,h) Mean fluorescence intensity of MHC class II (g) and TLR4 (h) in respective cell types. Lymphoid cells (gated with the forward/side scatters), B cells ($B220^+$), and myeloid cells ($CD11b^+$) were determined by flow cytometry. See Supplementary Figs. 1 and 2 for cell definition. Data are shown as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, based on Tukey's multiple comparisons test following a one-way ANOVA.

were significantly enriched were listed in Fig. 2b and c,3b-d,7c,8c,e.

2.7. Cell types enrichment analysis

Cell type enrichment analysis using xCell (version 1.1.0)[39] was conducted on transcriptome data from bone marrow and spleen. Due to xCell's design for analyzing human genes, a conversion from mouse to human genes was necessary, using homologous gene lists referenced from Ensemble BioMart (http://www.ensembl.org/biomart/martview/a27b4aeae56c7a4e281d8b9045100aa2). For this analysis, 13,979 genes from the bone marrow and 13,785 from the spleen were selected. Enrichment scores for each cell type, which was defined by xCell algorithm, were calculated, and those falling below the 25th percentile were not included in the visualization.

2.8. Flow cytometry analysis

Flow cytometry was performed as previously described.[13] Cell suspension from the bone marrow and spleen was incubated with anti-CD16/CD32 antibody (clone 2.4G2, TONBO Biosciences, RRID: AB_2621487) for Fc receptor block and then incubated with fluorescently labelled antibodies for 30 min at 4 °C, as follows: BV421-conjugated anti-mouse CD3e (clone 145-2C11, BioLegend; RRID: AB_11203705), APC-conjugated anti-mouse CD4 (clone RM4-5, BioLegend; RRID: AB_312718), PE/Dazzle 594-conjugated anti-mouse ST2 (clone DIH9, BioLegend; RRID: AB_2687365), FITC-conjugated anti-mouse CD25 (clone PC61.5, TONBO biosciences; RRID: AB_2621685), FITC-conjugated anti-mouse CD3e (clone 145-2C11, BioLegend; RRID: AB_312671), PE-conjugated anti-mouse CD8a (clone 53–6.7, BioLegend;

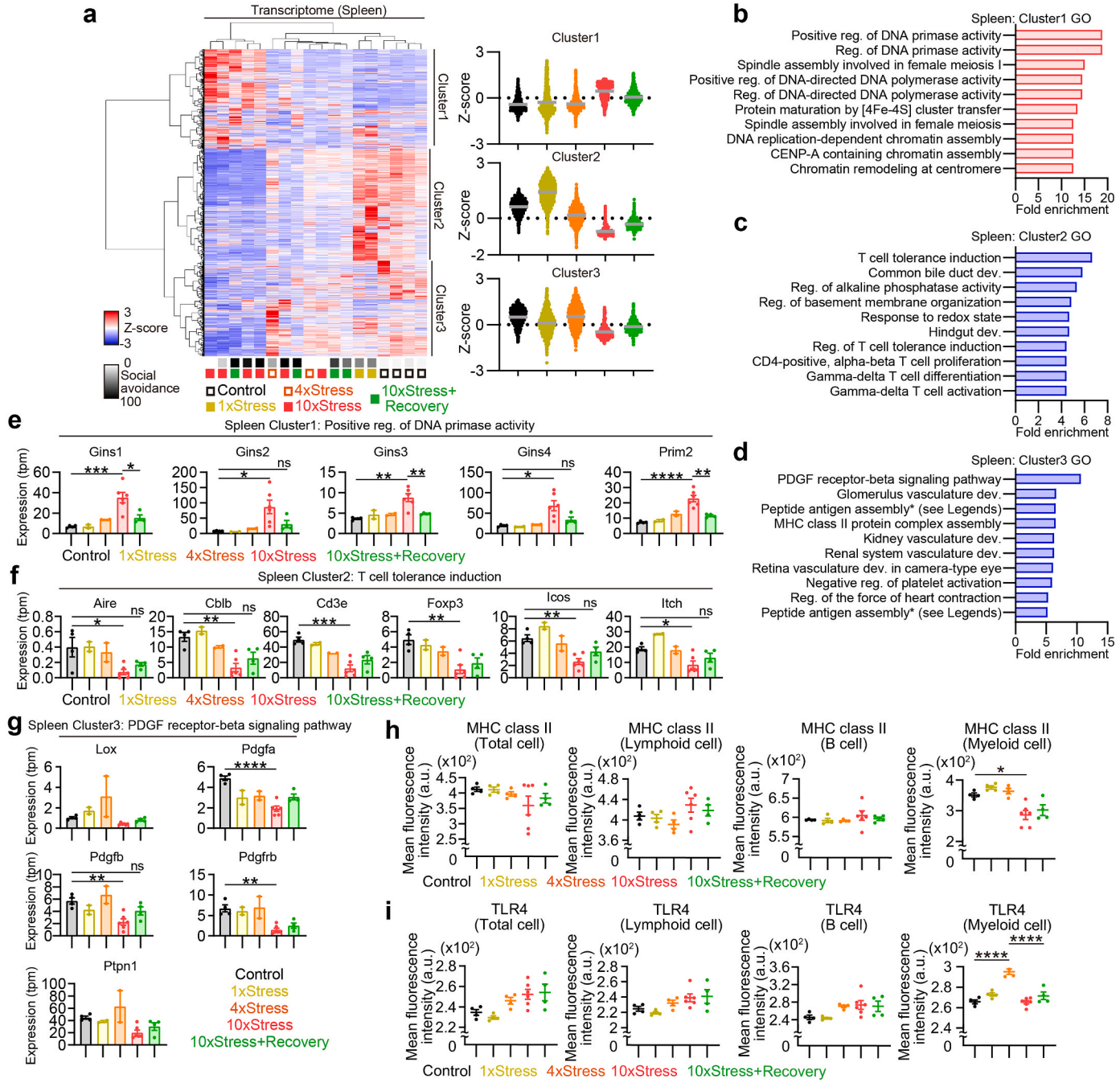

Fig. 3. Dysregulation of Immune-Related Gene Expression in the Spleen due to Chronic Stress

(a) Cluster analysis in the spleen transcriptome. The heat map (left) and bar graphs (right) illustrate Z-scores for genes in Clusters 1, 2, and 3. Below, mouse groups and their social avoidance levels are presented. Genes in Cluster 1 show upregulation during chronic stress, whereas those in Clusters 2 and 3 show downregulation. (b–d) Gene Ontology terms for Clusters 1, 2, and 3. These include terms linked to genes in Cluster 1 (b), Cluster 2 (c), and Cluster 3 (d), associated with statistically significant differences and a fold change of >2 or <0.5 when comparing the 10xStress and Control groups. "Reg." and "Dev." represent Regulation and Development. In panel d, the third and tenth entries are "Peptide antigen assembly with MHC class II protein complex" and "Peptide antigen assembly with MHC protein complex," respectively. (e–g) Gene expression levels in selected Gene Ontology terms. This shows the expression levels of genes within the terms "Positive regulation of DNA primase activity" (e), "T cell tolerance induction" (f), and "PDGF receptor-beta signaling pathway" (g) from the lists in (b–d). (h,i) Mean fluorescence intensity of MHC class II (h) and TLR4 (i) in respective cell types. Lymphoid cells (gated with the forward/side scatters), B cell ($B220^+$), and myeloid cell ($CD11b^+$) were determined by flow cytometry. Data are shown as means ± SEM. ns, not significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, based on Tukey's multiple comparisons test following a one-way ANOVA.

RRID: AB_312746), PE-conjugated mouse/human CD45R/B220 (clone RA3-6B2, BioLegend; RRID: AB_312992), BV421 anti-mouse CD11b (clone M1/70, BioLegend; RRID: AB_2562904), APC-conjugated anti--mouse F4/80 (clone BM8, BioLegend; RRID: AB_893493), PerCP-Cy5.5-conjugated anti-mouse Ly-6C (clone HK1.4, BioLegend; RRID: AB_1659242), FITC-conjugated anti-mouse Ly-6G (clone 1A8, BioLegend; RRID: AB_1236488), PE-conjugated anti-mouse CD117 (c-Kit) (clone 2B8, BioLegend; RRID: AB_313216), APC-conjugated anti-mouse CD49b (clone DX-5, BioLegend; RRID: AB_313416), FITC-conjugated anti-mouse FcεRI (clone MAR-1, BioLegend; RRID:

AB_1626102), APC-conjugated mouse/human CD45R/B220 (clone RA3-6B2, BioLegend; RRID: AB_312996), PE-conjugated anti-mouse TLR4 (clone UT41, eBioscience; RRID: AB_466236), and FITC-conjugated anti-mouse MHC classII(clone M5/114.15.2, RRID: AB_465232). After incubation with the antibodies, erythrocytes were lysed with red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM EDTA). The remaining cells were counted with a hemocytometer, washed twice with D-PBS (−) containing 2 mM EDTA and 0.5% bovine serum albumin, and subjected to flow cytometry analysis. Fluorescent signals were detected and quantified using the CytoFLEX S system (Beckman Coulter, Brea, CA, USA).

Data were analyzed using FlowJo software (FlowJo LLC, BD Biosciences) with gating strategies shown in Supplementary Figs. 1 and 2. We used the forward scatter height and width to gate single cells, and then the forward and side scatter areas to gate viable cells, as previously described.[13] Among the viable cells, lymphoid cells were gated with the forward and side scatter areas. Then, B cells ($B220^+$), T cells ($CD3e^+$), $CD4^+$ T cells ($CD3e^+CD4^+$), Th2 cells ($CD3e^+CD4^+ST2^+$), $CD8^+$ T cells ($CD3e^+CD8a^+$), and regulatory T cells ($CD3e^+CD4^+CD25^+$) were gated with the designated markers. Among the viable cells, neutrophils ($CD11b^+Ly\text{-}6G^+$), macrophages ($CD11b^+F4/80^+$), monocytes ($CD11b^+Ly\text{-}6G^-Ly\text{-}6C^+$), and basophils ($CD11b^+CD117$ $(c\text{-}kit)^-CD49b^+Fc\varepsilon RI^+$) were gated by the designated markers. The mean fluorescent intensity for MHC class II and TLR4 among total cells and within specific cell types of interest was measured to assess their surface expression.

2.9. Statistical analyses

Data are presented as means ± SEM. Statistical analyses were conducted using Prism 10.0 software (GraphPad Software, San Diego, CA, USA). P-values below 0.05 were considered statistically significant, unless specified otherwise in the text. Forcomparing values across two independent factors, we utilized two-way ANOVA followed by Tukey's multiple comparisons test (see Fig. 6a–c). In cases of one independent factor, we applied one-way ANOVA, also followed by Tukey's multiple comparisons test (see Fig. 2d–h,3e-i,4b-f,5b-f,6b). For analyzing correlations, Pearson's correlation test was performed (see Figs. 7 and 8).

3. Results

3.1. Chronic stress induces distinct immune-related gene expression changes in the bone marrow and spleen

To investigate the effects of chronic stress on immune functions, we analyzed transcriptome and lipidome changes in femur bone marrows and spleens under various stress conditions. These conditions included periods before stress exposure, immediately after the first, fourth, and tenth exposures, and following a one-week recovery from the final exposure. We conducted RNA-seq and LC-MS/MS analyses on these tissue samples (Fig. 1a and b). Additionally, we assessed social avoidance, a common behavioral outcome of chronic stress, at these intervals. Our findings indicated that social avoidance progressively intensified, peaking after the tenth exposure (Fig. 1c and d).

In the bone marrow, a clustering analysis of detectable genes (17,227 genes) revealed two distinct groups: one with genes downregulated and another with genes upregulated due to chronic stress (Cluster 1 and Cluster 2, respectively) (Fig. 2a). These genes varied gradually during chronic stress, peaked after the tenth exposure, and partially reverted to normal post-recovery. Note that these fluctuations did not correlate with the level of social avoidance (Fig. 2a). Gene Ontology analysis identified that the downregulated genes in Cluster 1 were linked to adaptive immune responses, including MHC class II-mediated antigen presentation and regulation of gamma-delta T cell differentiation and activation. Conversely, upregulated genes in Cluster 2 correlated with innate immune responses, such as response to bacterial molecules and regulation of phagocytic processes (Fig. 2b and c). Within the MHC class II category, some genes were downregulated post-stress but returned to baseline post-recovery (Fig. 2d), while others remained downregulated even after recovery. Genes involved in lipopolysaccharide detection and neutrophil extravasation were upregulated post-stress but normalized post-recovery (Fig. 2e and f). These observations imply that chronic stress weakens adaptive immune responses in the bone marrow, altering T cell antigen repertoire post-recovery, whereas innate immune responses are induced during stress and resolved post-recovery.

Similarly, in the spleen, gene clustering (16,359 genes) identified three clusters: Cluster 1 with genes upregulated after chronic stress, Cluster 2 with genes fluctuating between acute and chronic stress, and Cluster 3 with genes downregulated after chronic stress (Fig. 3a). Like the bone marrow, gene expression changes in the spleen were more influenced by stress duration than social avoidance levels. Gene Ontology analysis revealed enrichment in DNA replication and chromatin assembly genes in Cluster 1, T cell tolerance induction in Cluster 2, and vascular development and antigen presentation in Cluster 3 (Fig. 3b–d). Genes associated with DNA primase activity were upregulated only after chronic stress, returning to baseline post-recovery (Fig. 3e). Genes related to T cell tolerance induction were downregulated post-stress, partially reverting post-recovery (Fig. 3f). Genes in the PDGF receptor-beta signaling pathway, which is involved in vascular development, followed a similar pattern (Fig. 3g). These findings indicate that chronic stress impairs T cell tolerance and vascularization in the spleen.

Collectively, these findings indicate that the bone marrow and spleen show distinct immune-related gene expression changes after chronic stress.

Using flow cytometry, we assessed the surface expression of representative genes, MHC class II and TLR4, which were affected by chronic stress in transcriptome analyses. In the bone marrow, consistent with mRNA expression, surface MHC class II expression was decreased in total and myeloid cells, although it was less affected in lymphoid and B cells (Fig. 2g). Although chronic stress increased TLR4 mRNA expression, surface TLR4 expression in total cells appeared to be decreased (Fig. 2h), suggesting that the protein level and/or surface presentation of TLR4 is regulated independently from its mRNA level. Among cell types, surface TLR4 expression was decreased in myeloid cells, whereas it was increased in lymphoid cells (Fig. 2h). In the spleen, surface MHC class II expression was decreased in myeloid cells, although no significant changes were observed in total cells (Fig. 3h). Surface TLR4 expression appeared to be increased in total cells, though not significantly. Among cell types, myeloid cells showed a transient peak of surface TLR4 expression at 4x Stress, whereas lymphoid and B cells showed no significant increase (Fig. 3i). These findings suggest that chronic stress attenuates antigen presentation capacity of myeloid cells in both the bone marrow and spleen, whereas TLR4 signaling is regulated in a tissue- and cell type-specific manner.

3.2. Chronic stress induces a shift from lymphoid to myeloid cells in the bone marrow and spleen with distinct temporal profiles

To estimate cell types responsible for these gene expression changes, we used the xCell algorithm for cell type enrichment analysis with transcriptome data from the bone marrow and spleen. In the bone marrow, chronic stress decreased the enrichment of gene signature for naïve and memory B cells and erythrocytes, while increasing that for myeloid cells such as macrophages, monocytes, and neutrophils (Fig. 4a). The enrichment of gene signature for endothelial cells, megakaryocytes, and platelets were largely unaffected during acute and chronic stress but increased post-recovery. Regarding hematopoietic cell differentiation, the enrichment of gene signature for hematopoietic stem cells (HSC) increased during stress but normalized post-recovery, while that for multipotent progenitor cells (MPP) was upregulated only after stress cessation. Enrichment of gene signature for common lymphoid

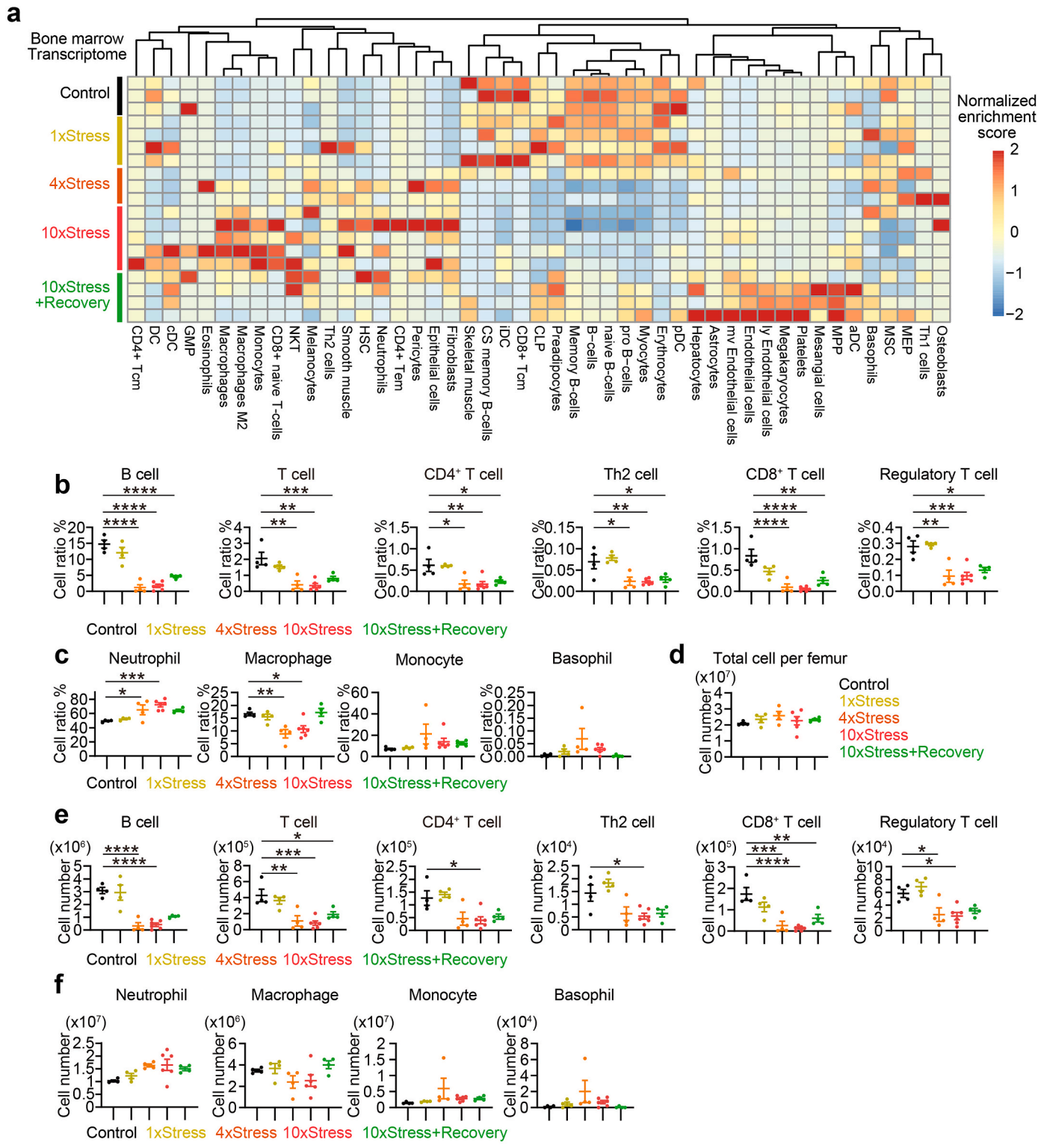

Fig. 4. xCell- and Flow Cytometry-Based Analysis of Cellular Composition: Revealing Chronic Stress-Induced Alterations in Immune Cell Profiles within Bone Marrow

(a) Cell types enrichment analysis was conducted using transcriptome data from the bone marrow. The color encoding reflects Z-scores calculated from the enrichment score of each cell type. Cell types with enrichment scores below the 25th percentile were not included in the visualization. Tcm, central memory T-cells; DC, dendritic cells; cDC, conventional DC; GMP, granulocyte-macrophage progenitors; NKT, natural killer T-cells; Th2, Type 2 T-helper; HSC, hematopoietic stem cells; Tem, effector memory T-cells; CS, class-switched; iDC, immature DC; CLP, common lymphoid progenitors; pDC, plasmacytoid DC; mv, microvascular; ly, lymphatic; MPP, multipotent progenitors; aDC, activated DC; MSC, mesenchymal stem cells; MEP, megakaryocyte-erythroid progenitors; Th1, type 1 T-helper. (b–f) Relative abundance (b,c) and absolute number (e,f) of each cell type as well as the total cell number (d) in a femur bone of each mouse, determined by flow cytometry analysis. Data are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, based on Tukey's multiple comparisons test following a one-way ANOVA. See Supplementary Figs. S1 and S2 for gating strategies in flow cytometry analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

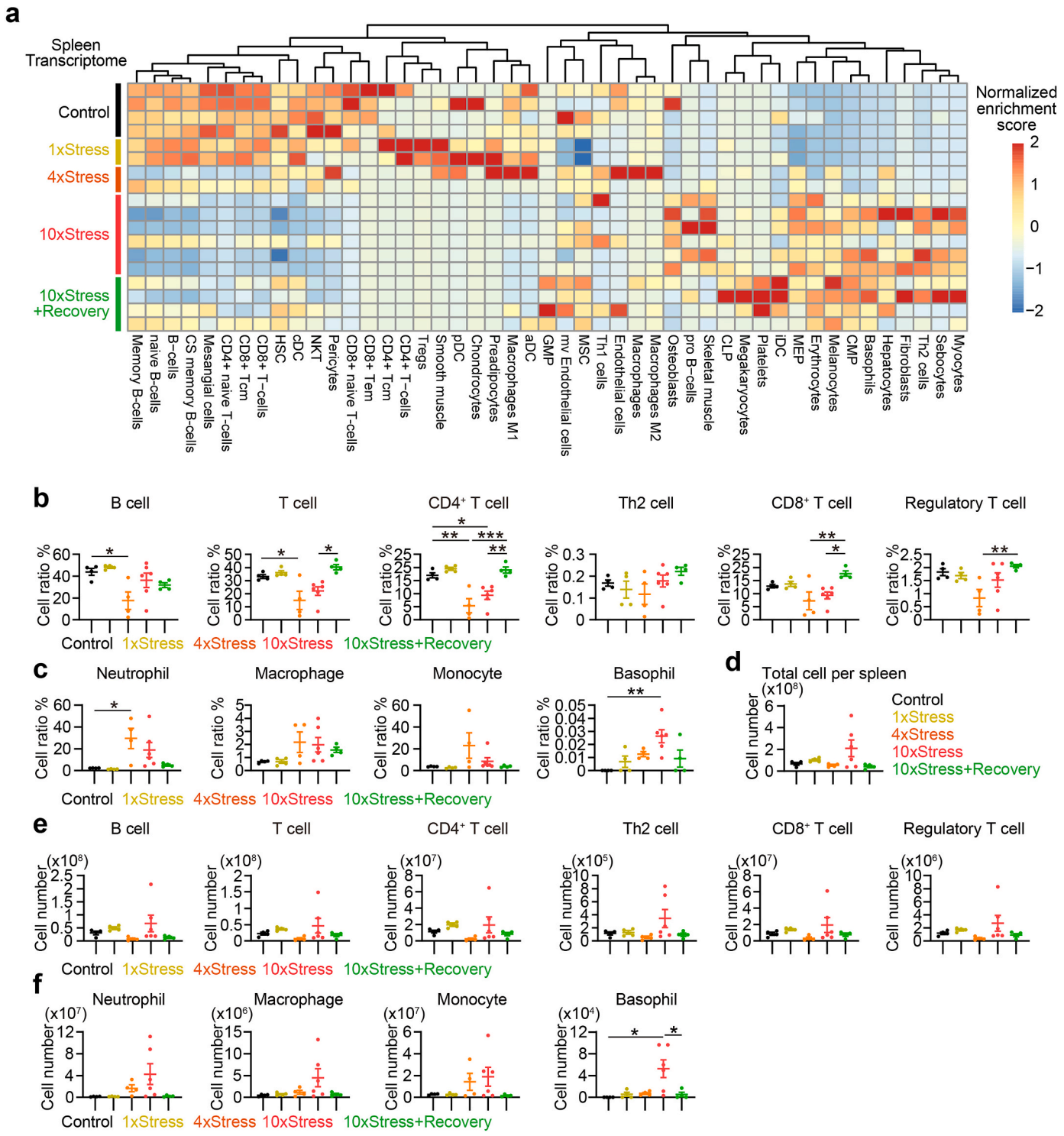

Fig. 5. xCell- and Flow Cytometry-Based Analysis of Cellular Composition: Revealing Chronic Stress-Induced Alterations in Immune Cell Profiles within Spleen
(a) Cell types enrichment analysis was conducted using transcriptome data from the bone marrow. The color encoding reflects Z-scores calculated from the enrichment score of each cell type. Cell types with enrichment scores below the 25th percentile were not included in the visualization. CS, class-switched; Tcm, central memory T-cells; HSC, hematopoietic stem cells; DC, dendritic cells; cDC, conventional DC; NKT, natural killer T-cells; Tem, effector memory T-cells; Tregs, regulatory T-cells; pDC, plasmacytoid DC; aDC, activated DC; GMP, granulocyte-macrophage progenitors; mv, microvascular; MSC, mesenchymal stem cells; Th1, type 1 T-helper; CLP, common lymphoid progenitors; iDC, immature DC; MEP, megakaryocyte-erythroid progenitors; CMP, common myeloid progenitors; Th2, Type 2 T-helper. (b–f) Relative abundance (b,c) and absolute number (e,f) of each cell type as well as the total cell number (d) in the spleen of each mouse, determined by flow cytometry analysis. Data are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, based on Tukey's multiple comparisons test following a one-way ANOVA. See Supplementary Figs. S1 and S2 for gating strategies in flow cytometry analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

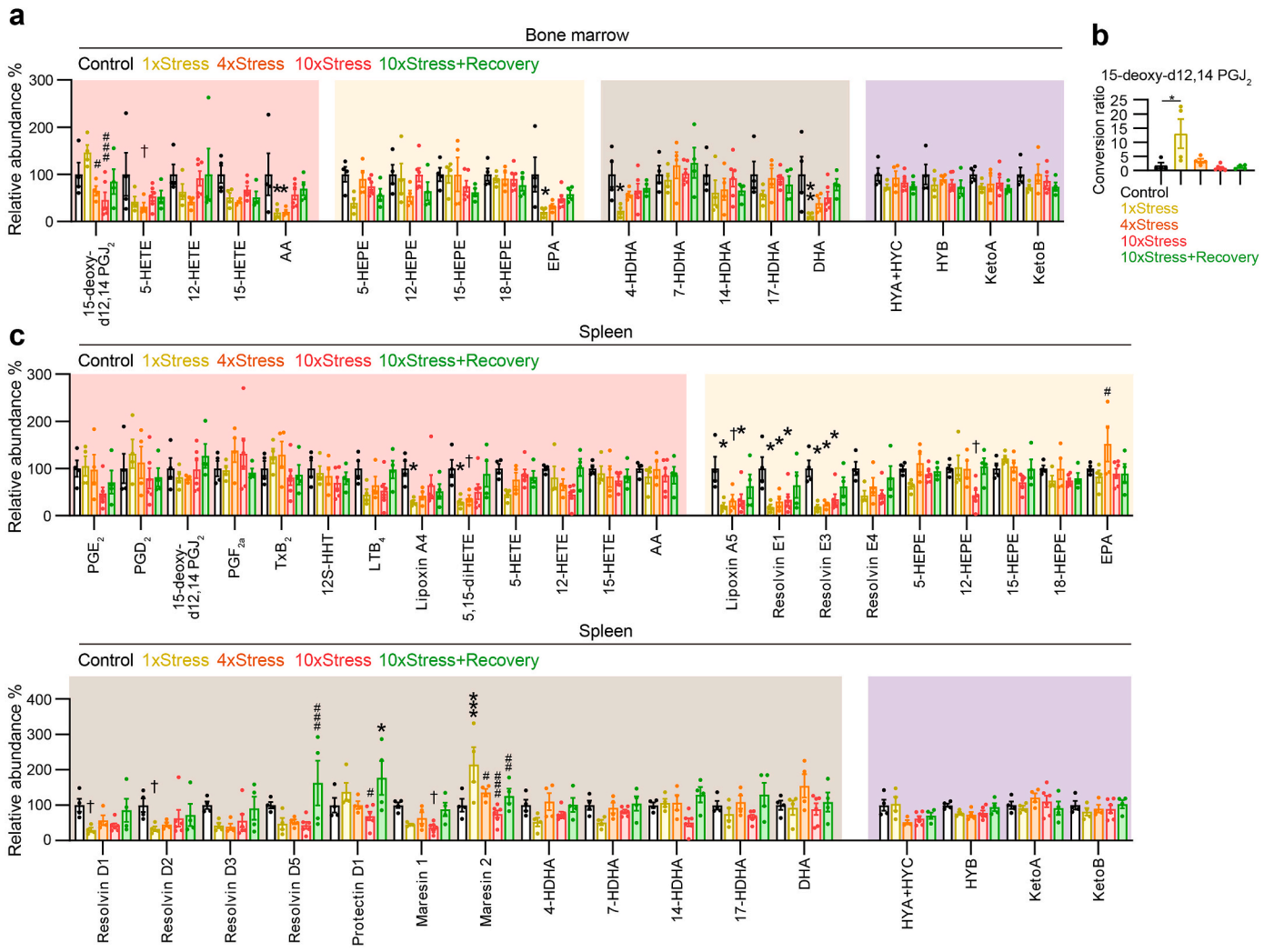

Fig. 6. Distinct Effects of Chronic Stress on the Lipid Mediator Profiles in Bone Marrow and Spleen
(a,c) Quantification of lipid mediators in bone marrow and spleen. The relative concentrations of detectable lipid mediators in bone marrow and spleen are shown, respectively. The metabolites associated with arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and linoleic acid are illustrated against pink, yellow, beige, and purple backgrounds, correspondingly. These values are normalized relative to Control mice and presented as means ± SEM. Statistical significance is denoted by †$P < 0.1$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, based on Tukey's multiple comparisons test, following a two-way ANOVA, to contrast the Control group with each Stress group. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, in comparisons between the 1xStress and other Stress groups. (b) Conversion ratio of 15-deoxy-d12,14 PGJ_2 and AA. The conversion ratio between the relative abundance of 15-deoxy-d12,14 PGJ_2 and its precursor AA is shown. *P < 0.05, based on Tukey's multiple comparisons test following a one-way ANOVA. PG; Prostaglandin, Tx; Thromboxane, LT; Leukotriene, HETE; Hydroxyeicosatetraenoic acid, HEPE; Hydroxyeicosapentaenoic acid, HDHA; hydroxy DHA, HYA; 10-hydroxy-cis-12-octadecenoic acid, HYC; 10-hydroxy-trans-11-octadecenoic acid, HYB; 10-hydroxyoctadecanoic acid, KetoA; 10-oxo-cis-12-octadecenoic acid, KetoB; 10-oxooctadecanoic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

progenitor cells (CLP) and megakaryocyte-erythroid progenitor cells (MEP) decreased during stress, whereas that for granulocyte-monocyte progenitor cells (GMP) was unaffected.

In the spleen, chronic stress decreased the enrichment of gene signature for both naïve and memory B cells, as well as naïve and memory T cells (Fig. 5a). In contrast, the enrichment of gene signature for other cell types, such as erythrocytes, basophils, Th2 cells, and fibroblasts, increased. These changes persisted during a one-week recovery period. Acute stress did not affect the enrichment of gene signature for these cells, but it transiently increased that for $CD4^+$ T cells, regulatory T cells, and smooth muscle cells. The enrichment of gene signature for megakaryocytes and platelets remained stable during chronic stress but was upregulated after a week of recovery. Regarding hematopoietic cell differentiation, chronic stress reduced the enrichment of gene signature for HSC and elevated that for CMP, MEP, and pro-B cells. The enrichment of gene signature for GMP and CLP was unaffected during chronic stress but increased after one-week recovery.

We then validated xCell-based cell population estimates using flow cytometry. In the bone marrow, chronic stress decreased the relative abundance of B cells, T cells (including $CD4^+$ T cells, Th2 cells, $CD8^+$ T cells, and regulatory T cells), and macrophages and increased those of neutrophils (Fig. 4b and c). The decrease in B and T cells persisted after one week of recovery. These changes in B cells and neutrophils confirmed xCell-based estimates, although xCell was not sensitive enough to detect the changes in other cell types. Given that the total cell number per femur was unaltered, the absolute numbers of respective cell types recapitulated the results, although the neutrophil increase was not statistically significant (Fig. 4d–f).

In the spleen, chronic stress decreased the relative abundance of B and T cells and increased those of neutrophils and basophils, mostly consistent with the xCell-based estimates (Fig. 5b and c). These changes were transient, returning to baseline after one week of recovery, unlike

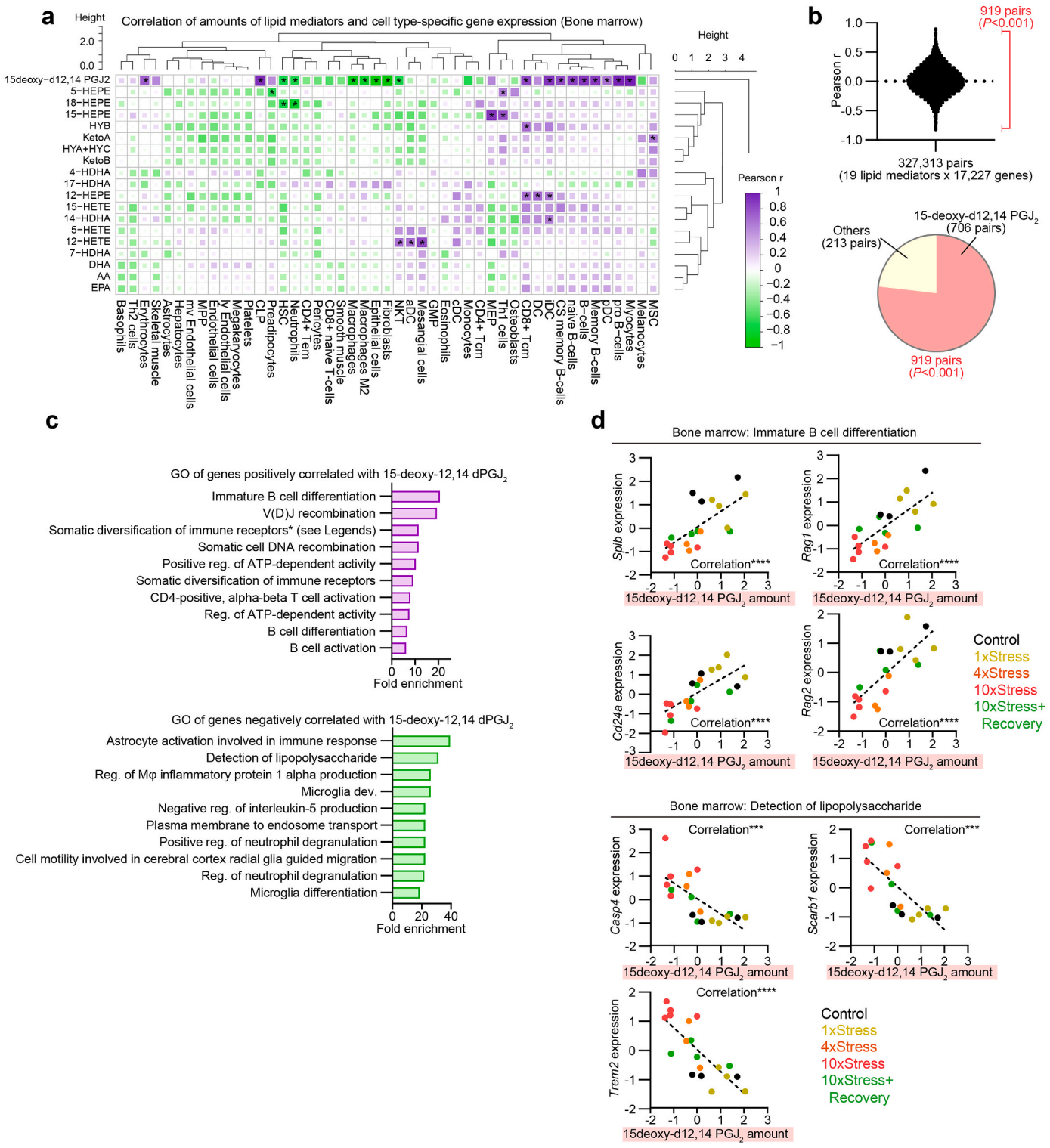

Fig. 7. Correlation of 15-Deoxy-d12,14-Prostaglandin J_2 with Gene Expressions in Bone Marrow

(a) Correlation matrix between cell type enrichment scores and lipid mediator amounts in bone marrow. 15-deoxy-d12,14 PGJ_2 demonstrates both positive and negative correlations with the enrichment of gene signature for diverse immune-related cell types. The purple and green colors in each cell represent positive and negative correlation coefficients (r), respectively. The size of each colored box indicates the -log10(p) value from Pearson correlation analysis, with asterisks denoting statistical significance ($P < 0.05$). (b) Correlation coefficients between lipid mediators and gene expressions in bone marrow. This analysis shows the correlation coefficients for all combinations of lipid mediators (19 species) and gene expressions (17,227 genes). Out of 327,313 pairs, 919 were found to be statistically significant with $P < 0.001$. Notably, 706 of these significant pairs are associated with 15-deoxy-d12,14 PGJ_2. (c) Gene Ontology terms for genes associated with 15-deoxy-d12,14 PGJ_2. This illustrates Gene Ontology terms linked to genes that are either positively or negatively correlated with the amount of 15-deoxy-d12,14 PGJ_2. (d) Correlation analysis for selected Gene Ontology terms. This highlights the correlation between the amount of 15-deoxy-d12,14 PGJ_2 and the expression levels of genes within specific Gene Ontology terms, "Immature B cell differentiation" and "Detection of lipopolysaccharide". *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, based on Pearson's correlation test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

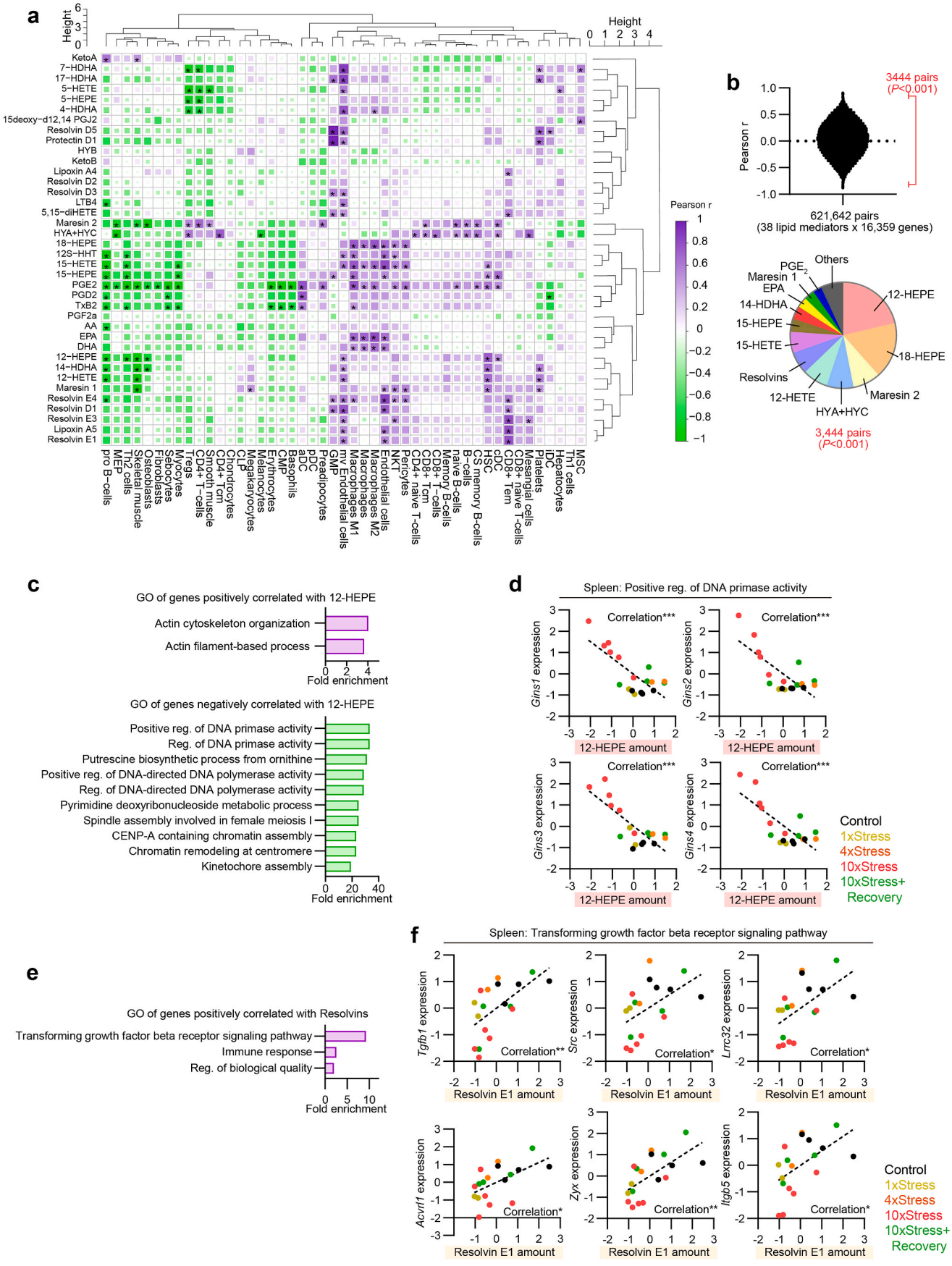

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Fig. 8. Correlation of 12-HEPE and resolvins with Gene Expressions in Spleen
(a) Correlation matrix between cell type enrichment scores and lipid mediator amounts in spleen. The purple and green colors in each cell represent positive and negative correlation coefficients (r), respectively. The size of each colored box indicates the -log10(p) value from Pearson correlation analysis, with asterisks denoting statistical significance ($P < 0.05$). (b) Correlation coefficients between lipid mediators and gene expressions in spleen. This analysis shows the correlation coefficients for all combinations of lipid mediators (38 species) and gene expressions (16,359 genes). Out of 621,642 pairs, 3444 were found to be statistically significant with $P < 0.001$. Notably, 734 and 235 of these significant pairs are associated with 12-HEPE and resolvins, respectively. (c,e) Gene Ontology terms for genes associated with 12-HEPE (c) and resolvins (e). This illustrates Gene Ontology terms linked to genes that are either positively or negatively correlated with the amount of 12-HEPE and resolvins. (d,f) Correlation analysis for selected Gene Ontology terms. This highlights the correlation between the amount of 12-HEPE (d) and resolvins (f) and the expression levels of genes within specific Gene Ontology terms, "Positive regulation of DNA primase activity" and "Transfoming growth factor beta receptor signaling pathway". $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, based on Pearson's correlation test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the bone marrow. However, since the spleen was enlarged with the total cell number increased (Fig. 5d), the decrease in B and T cells was masked in their absolute number (Fig. 5e). The absolute numbers of basophils and neutrophils appeared to be increased, although statistical significance was not reached for the latter (Fig. 5f).

Collectively, these findings revealed that chronic stress induces a shift from lymphoid to myeloid cells in the bone marrow and spleen, exhibiting distinct temporal profiles.

3.3. Acute and chronic stress differentially regulate anti-inflammatory and pro-resolving lipid mediators in bone marrow and spleen

We subsequently conducted an analysis of lipid metabolite levels in both the bone marrow and spleen under various stress conditions. In the bone marrow, acute stress led to a reduction in lipid mediator precursors such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), with levels partially reverting to baseline following chronic stress (Fig. 6a–Supplementary Table 1). Among the lipid mediators that were detectable, while the majority remained largely unchanged, significant changes were observed in 15-deoxy-d12,14 PGJ_2 and 4-HDHA. Specifically, 15-deoxy-d12,14 PGJ_2 levels appeared to increase following acute stress and decrease after chronic stress, diverging from the pattern of its precursor AA. The ratio of the amount of 15-deoxy-d12,14 PGJ_2 to that of its precursor AA (i.e., conversion ratio), which reflects this metabolic processing efficiency, suggests that acute stress temporarily enhanced the synthesis of 15-deoxy-d12,14 PGJ_2 from AA (Fig. 6b). Conversely, 4-HDHA levels decreased under acute stress and only partially recovered following chronic stress, mirroring the pattern of its precursor DHA (Fig. 6a). Given that both lipid mediators are known to play anti-inflammatory roles, in part by binding to PPARγ, these results imply that acute and chronic stress differentially regulates inflammatory response by modulating anti-inflammatory lipid mediators in the bone marrow.

In the spleen, neither acute nor chronic stress had a significant impact on lipid mediator precursors such as AA, EPA, and DHA (Fig. 6c–Supplementary Table 2). Among the lipid mediators that could be detected, many pro-resolving lipid mediators (e.g., lipoxin A4, lipoxin A5, resolvin E1, resolvin E3, resolvin D1, resolvin D2) decreased in response to acute stress. This reduction persisted during chronic stress for most of these mediators and partially reverted to baseline during a one-week recovery period. However, maresin 2, a pro-resolving lipid mediator, exhibited a transient increase during acute stress and then returned to baseline after one week of recovery. Additionally, 12-HEPE, an anti-inflammatory lipid mediator that influences cellular metabolism, only decreased following chronic stress. These observations suggest that both acute and chronic stress prolong inflammation in the spleen by inhibiting the production of anti-inflammatory and pro-resolving lipid mediators.

Although we sought to identify the synthesizing and/or degrading enzymes associated with chronic stress-induced changes in lipid mediators, the expression levels of these enzymes were not changed in a manner correlated to the levels of lipid mediators. Similar to the findings in the transcriptome analysis, we found no significant correlations between lipid mediator levels and social avoidance, suggesting that the quantity of lipid mediators is determined by stress duration rather than its effect on social behavior.

Taken together, these findings reveal that acute and chronic stress distinctly regulate anti-inflammatory and pro-resolving lipid mediators in the bone marrow and spleen.

3.4. Stress-induced lipid mediator changes correlate with specific immune responses in bone marrow and spleen

To examine the connection between changes in lipid mediators and those in immune cell populations and transcriptomes, we analyzed how lipid mediator levels correlate with xCell-based enrichment scores and gene expression levels.

In the bone marrow, among stress-affected lipids, the precursors (such as AA, EPA, DHA) did not show significant correlations with the enrichment of gene signature for any cell types. However, 15-deoxy-d12,14 PGJ_2 exhibited positive correlations with the enrichment of gene signature for lymphoid cells and their precursors (including naïve and memory B cells, pro B-cells, central memory $CD8^+$ T cells, CLP) and erythrocytes, and negative correlations with that for myeloid cells (such as macrophages, neutrophils) and others (including HSC, NKT, epithelial cells, fibroblasts). We further assessed the correlation coefficient and statistical significance using Pearson's correlation analysis across all 327,313 pairs of detectable lipid mediators (19 species) and detectable gene expressions (17,227 genes). Conventional P-value thresholds of 0.05 and 0.01 yielded marginally more pairs than expected by chance. Therefore, we set a more stringent threshold of 0.001, which resulted in 919 pairs exceeding the chance level ($0.001 \times 327{,}313 = 327$ pairs) (Fig. 7b). Of these, the highest number of gene associations was found for 15-deoxy-d12,14 PGJ_2 (706 pairs, 76.8%). Gene Ontology analysis indicated that genes positively correlated with 15-deoxy-d12,14 PGJ_2 were linked to B cell differentiation and activation, including V(D)J recombination and somatic diversification of antibodies (Fig. 7c and d). In contrast, genes negatively correlated with 15-deoxy-d12,14 PGJ_2 were associated with innate immune responses, like lipopolysaccharide detection and myeloid cell development and functions. These results suggest that in the bone marrow, a chronic stress-induced decrease in 15-deoxy-d12,14 PGJ_2 corresponds to changes in gene expressions leading to a reduction in B cells and an increase in myeloid cells.

In the spleen, among stress-affected lipids, most pro-resolving lipid mediators that decreased after acute and chronic stress (such as lipoxin A5, resolvin E1, resolvin E3, resolvin D1) correlated positively with the enrichment of gene signature for effector memory $CD8^+$ T cells and endothelial cells (Fig. 8a). Maresin-2, which increased transiently after acute stress, showed a positive correlation with the enrichment of gene signature for other adaptive immune cells. 12-HEPE, which decreased only after chronic stress, correlated positively with the enrichment of gene signature for HSC and cDC, and negatively with that for Th2 cells, which increased only after chronic stress. We then analyzed the correlation coefficient and statistical significance using Pearson's correlation with all 621,642 pairs between detectable lipid mediators (38 species) and detectable gene expressions (16,359 genes). Using the stringent P value threshold of 0.001 for statistical significance, we identified 3444 pairs exceeding the chance level ($0.001 \times 621{,}642 = 621$ pairs)

(Fig. 8b). The highest number of gene associations was observed for 12-HEPE (734 pairs, 21.3%), 18-HEPE (591 pairs, 17.2%), maresin 2 (290 pairs, 8.4%), HYA + HYC (282 pairs, 8.2%), 12-HETE (266 pairs, 7.7%), and resolvins (235 pairs, 6.8%). Gene Ontology analysis revealed that genes positively correlated with 12-HEPE were linked to the regulation of the actin cytoskeleton (Fig. 8c). Conversely, genes negatively correlated with 12-HEPE were associated with DNA replication and chromatin assembly (Fig. 8c and d), the most enriched Gene Ontology terms for genes upregulated after chronic stress. This analysis also indicated that genes positively correlated with resolvins were linked to the TGF-β signaling pathway, known for its anti-inflammatory roles (Fig. 8e and f). These findings imply that in the spleen, a chronic stress-induced decrease in 12-HEPE and resolvins is related to gene expressions involved in extramedullary hematopoiesis and prolonged inflammation.

In summary, these results demonstrate that the regulation of anti-inflammatory and pro-resolving lipid mediators is closely connected with specific innate and adaptive immune responses triggered by acute and chronic stress in both the bone marrow and spleen.

4. Discussion

In our study, we explored the effects of acute and chronic stress, as well as their recovery, on immune-related gene expression, cellular composition, and lipid mediators in the bone marrow and spleen. In the bone marrow, chronic stress induced a sustained transition from lymphoid to myeloid cells, accompanied by corresponding changes in gene expression. This stress also reduced levels of 15-deoxy-d12,14 PGJ_2, a lipid mediator known to suppress inflammation partially via PPARγ activation. This reduction correlated with changes in gene expression that favored a decrease in B cells (linked to B cell differentiation and activation, as highlighted by GO terms) and an increase in myeloid cells (associated with innate immune responses, as per GO terms). In the spleen, chronic stress also induced a lymphoid-to-myeloid transition, albeit transiently, alongside gene expression changes indicative of extramedullary hematopoiesis. It lowered levels of 12-HEPE and resolvins, both key in suppressing and resolving inflammation.[40,41] The decrease in these mediators was linked to gene expressions aligned with extramedullary hematopoiesis (related to DNA replication and chromatin assembly, as indicated by GO terms) and prolonged inflammation (associated with the TGF-β signaling pathway, as per GO terms), respectively. Our findings indicate that chronic stress prompts tissue-specific immune responses in the bone marrow and spleen by differentially modulating various innate and adaptive immune cells, partly through the regulation of anti-inflammatory and pro-resolving lipid mediators.

Although we identified chronic stress-induced changes in immune-related gene expressions and lipid mediators, no correlations were found with the degree of social avoidance. It should be noted that chronic stress induces not only social avoidance but also other behavioral changes, such as anhedonia, increased anxiety, and cognitive deficits. Whereas the degree of social avoidance varies among individuals, some behavioral alterations, such as increased anxiety and impaired cognitive flexibility, are reportedly affected regardless of the degree of social avoidance.[3,42] Therefore, further study is warranted to investigate how changes in immune-related gene expressions, cell compositions, and lipid mediators induced by chronic stress relate to behavioral changes beyond social avoidance.

In this study, we unbiasedly estimated the effects of chronic stress on immune cell populations using xCell algorithm and validated the estimates with flow cytometry. Both analyses shared many changes in immune-related cell populations under chronic stress, such as decrease in B cells and increase in neutrophils in the bone marrow and decrease in B and T cells and increase in basophils in the spleen, findings that align with previous flow cytometry studies.[13,16,18,43,44] However, other changes, such as T cell decrease in the bone marrow and neutrophil increase in the spleen, were not detected with xCell algorithm. Thus, xCell-based estimation needs to be supplemented by the validation with flow cytometry. Nonetheless, the convergence of two independent methods on the same conclusion has reinforced the significance of the findings related to changes in immune-related cell populations induced by chronic stress in this study. Additionally, our xCell analysis showed an increase in the enrichment of gene signature for hematopoietic progenitors, such as CMPs and MEPs, and erythrocytes in the spleen. Although these changes were not validated by flow cytometry analysis in this study, they are consistent with the homing of hematopoietic progenitors and extramedullary hematopoiesis reported previously.[16] Our findings also pointed to a chronic stress-induced decrease in adaptive immune cells, particularly B and T cells, in the spleen, though previous studies on this effect have shown varied results.[45,46] The reason for this discrepancy is unclear, but it may be due to differences in stress conditions, such as type and duration of stressor and cage environment. A systematic analysis of the effects of chronic stress on adaptive immune cells in the spleen and other lymphoid tissues is still needed.

Gene expression changes have corroborated well-known effects of chronic stress, such as the lymphoid-to-myeloid shift. The combination of transcriptome, xCell and flow cytometry analyses have also uncovered novel immune functions of chronic stress. For instance, chronic stress reduced MHC class II expression in myeloid cells in the bone marrow, implying a decrease in antigen presentation of myeloid cells to T cells. After a one-week recovery, many MHC class II molecules remained reduced, indicating a lasting change in the antigen repertoire essential for cellular immunity. In the spleen, GO analysis identified chronic stress-induced gene expression changes related to vascular development, notably a reduction in PGDF receptors.[47] Furthermore, our findings shows basophil increase in the spleen due to chronic stress, potentially leading to increased susceptibility to allergic reactions.[48] Additionally, the xCell analysis showed an elevation in the enrichment of gene signature for megakaryocytes and platelets in both the bone marrow and spleen, but only after a week's recovery. This observation, although not validated by flow cytometry analysis, aligns with known stress-induced platelet activation in humans[49,50] and points to unexplored, post-stress, platelet-mediated processes. Confirming these hypotheses will require cell type-specific analyses and manipulations.

In our lipidome analysis, we identified certain lipid mediators whose levels were affected by both acute and chronic stress in the bone marrow and spleen. Acute stress in the bone marrow significantly lowered the levels of lipid mediator precursors such as AA, EPA, and DHA, with only a partial recovery observed under chronic stress. This suggests rapid turnover of these precursors and a stress-induced reduction in their synthesis. In contrast, the amount of 15-deoxy-d12,14 PGJ_2 was relatively maintained under acute stress and decreased under chronic stress. Conversely, the level of 4-HDHA decreased after acute stress and partially recovered during chronic stress. Since both mediators partly exert anti-inflammatory effects through PPARγ activation,[51,52] our findings suggest that acute and chronic stress differentially modulate inflammation through complex regulation of these mediators. In the spleen, levels of most lipid mediators from the resolvin D and E families were reduced under both stress conditions. Additionally, 12-HEPE levels declined after chronic stress. Given that these mediators have anti-inflammatory and pro-resolving roles, their alterations could lead to heightened and prolonged inflammation. This is consistent with the findings in this study as well as previous ones that chronic stress increases neutrophils and monocytes in the spleen.[13] Importantly, the stress-induced alterations in lipid mediators and their precursors differed between the bone marrow and spleen. Since the amounts of these lipid mediators were not correlated to the expression levels of the synthesizing and/or degrading enzymes, they could be regulated by protein levels of the enzymes and/or the regulation of their activity. Indeed, Alox5, an enzyme essential for synthesizing resolvin families, is regulated through phosphorylation and nuclear translocation.[53] Similarly, Alox12, which is responsible for 12-HEPE synthesis, can be phosphorylated, although the role of its phosphorylation remains

unknown.[54,55] Further research is necessary to elucidate the mechanisms underlying the alterations in lipid mediator levels in the bone marrow and spleen.

In this study, we have further clarified the connection between chronic stress-induced alterations in lipid mediators and corresponding changes in gene expressions and cell populations. However, since our analysis is purely correlational, the direct causal impact of lipid mediators on gene expressions, cell populations, and behaviors remains unclear. Previous research in both rodents and humans has indicated the significant roles of lipid mediators, particularly ω3-PUFAs and their metabolites, in the mobilization of leukocytes and in the development of depression and related behaviors.[28–30,37] It has been reported that dietary ω3-PUFAs affect their levels in the bone marrow, subsequently inhibiting sympathetic nerve-induced leukocyte mobilization from the bone marrow through PPARδ.[37] Consequently, a stress-induced reduction in ω3-PUFAs in the bone marrow may increase the stress-induced mobilization of myeloid cells, contributing to depression-related behaviors. Since splenectomy has been shown to eliminate the behavioral effects of prior stress exposure,[18] known as stress sensitization, where prior stress exposure exacerbates the effect of the following stress exposure, the depletion of resolvins in the spleen due to stress might play a role in this phenomenon by enhancing and prolonging inflammation in the spleen. Dietary ω3-PUFAs could potentially compensate for their deficiency in the bone marrow and the shortage of resolvins in the spleen caused by stress, thereby mitigating stress-induced leukocyte mobilization and its behavioral implications. Although infusing resolvins into the brain has been reported to alleviate depression-related behaviors caused by inflammation and chronic stress,[32] it is still uncertain whether endogenous resolvins are present and active in the brain, and if dietary ω3-PUFAs and their metabolites exert their behavioral effects via the brain. Therefore, the behavioral implications of changes in lipid mediators in the bone marrow and spleen merit further investigation. Prior studies have pinpointed specific enzymes responsible for producing each lipid mediator and receptors accountable for their functions. A deeper understanding of these enzymes and receptors will help elucidate the causal links between lipid mediators, immune-related changes in the bone marrow and spleen, and the behavioral effects of chronic stress.

Author contributions

I.H., H.N. and T.F. designed the study; I.H., H.N., M.T., G.C., M.S., T. S., S.I., Y.K., S.K. and T.F. performed and analyzed the results; I.H., H.N. and T.F. wrote the manuscript.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2024.02.010.

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