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Perforin Generated by CD8$^+$ T Cells Exacerbates Inflammatory Bowel Disease–Induced Depression by Promoting CXCL9 Production in Intestinal Epithelial Cells

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BACKGROUND & AIMS: Approximately 25.2% of patients with inflammatory bowel disease (IBD) suffer from psychological disorders, particularly depression. Recent studies have indicated a close relationship between intestinal immunity and brain disorders. **METHODS:** We performed transcriptome analysis and immunofluorescence staining of colonic samples from patients with IBD. The role of perforin generated by colonic CD8$^+$ T cells in IBD-induced depression was investigated in dextran sulfate sodium– and CD8$^+$ T-cell transfer-induced colitis by using *Prf1*-EGFP reporter and *Prf1* knockout mice. **RESULTS:** In this study, we revealed a significant correlation between depressive symptom severity and perforin production in CD8$^+$ T cells in both patients with IBD and mice with colitis. Moreover, perforin deficiency in CD8$^+$ T cells mitigated both inflammation and depressive-like behaviors in mice with colitis. Mechanistically, perforin and granzyme B were found to stimulate the expression of CXCL9 in colonic epithelial cells. CXCL9 was shown to be released into the circulation and to enter the hippocampus, where it induced endoplasmic reticulum stress in hippocampal neurons through the CXCR3-HSPA5 axis. This cascade of events subsequently was found to exacerbate depression. Neutralizing CXCL9 in vivo alleviated depression but had no effect on colitis in mice. **CONCLUSIONS:** Perforin generated by colonic CD8$^+$ T cells promotes intestinal epithelial cell CXCL9 production, which leads to neuronal endoplasmic reticulum stress in hippocampus and induces depression in IBD.

Keywords: Perforin; CD8$^+$ T Cells; Inflammatory Bowel Disease; Depression; CXCL9.

Inflammatory bowel disease (IBD) is a chronic and debilitating disease caused by inflammation of the gastrointestinal tract, including Crohn's disease and ulcerative colitis (UC). Patients with IBD may report symptoms associated with common mental disorders. Pooled prevalence of depression symptoms was 25.2%, and the prevalence of symptoms of depression was higher in patients with active IBD (38.9%) than in patients with inactive disease (24.2%).[1] Psychological illness further negatively affects disease outcomes and quality of life.[2] Numerous studies have revealed significant variability in the severity of depression among patients with IBD, but the mechanisms underlying this pronounced heterogeneity remain elusive. The intricate communication between the gut and the brain, termed the "gut-brain axis," might render patients with IBD particularly susceptible to the development of depression.[3]

The bidirectional link between T cells and depression is evident not only by the comorbidity of multiple T-cell–mediated immune diseases with depressive symptoms but also by the significant T-cell activation observed in patients with depression.[4,5] CD8$^+$ T-cell infiltration is positively correlated with the progression of IBD.[6] Synergizing with granzyme molecule, perforin is a classical effecter expressed by CD8$^+$ T cells, which results in the release of cytokines

Abbreviations used in this paper: **CSF, cerebrospinal fluid; DSS, dextran sulfate sodium; ER, endoplasmic reticulum; FBS, fetal bovine serum; GzmB, granzyme B; IBD, inflammatory bowel disease; IFN, interferon; PBS, phosphate-buffered saline; QIDS, Quick Inventory of Depressive Symptomatology; RNA-seq, RNA sequencing; SCCAI, Simple Clinical Colitis Activity Index; siRNA, small interfering RNA; UC, ulcerative colitis; WT, wild type.**

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Approximately 25.2% of patients with inflammatory bowel disease patients suffer from psychological disorders, particularly depression. However, the underlying mechanisms still remain unclear.

NEW FINDINGS

Our findings highlight the novel role of perforin produced by colonic $CD8^+$ T cells in inflammatory bowel disease–induced depression by promoting CXCL9 production in intestinal epithelial cells.

LIMITATIONS

In current study, all animal experiments were conducted using male mice exclusively and the potential impact of sex differences remains to be explored.

CLINICAL RESEARCH RELEVANCE

The identification of perforin and CXCL9 as key regulators of inflammatory bowel disease–induced depression opens up new possibilities for therapies.

BASIC RESEARCH RELEVANCE

This study contributes to a better understanding of the mechanisms involved in depression induced by inflammatory bowel disease.

and cell damage of target cells.[7] In the interaction between the immune and nervous systems, cytokines form a "bridge of communication." Several cytokines affect brain regions, particularly the hippocampus, which is a part of the limbic system associated with mood regulation and the dysfunction of which can lead to mental disorders.[8]

In the present study, we revealed that the production of perforin during colonic infiltration of $CD8^+$ T cells contributed to depressive symptoms in patients with UC and colitis model mice. Deficiency of perforin in $CD8^+$ T cells protected mice from colitis-induced depressive-like behaviors. Perforin and granzyme B (GzmB) generated by $CD8^+$ T cells stimulated the expression of CXCL9 in intestinal organoids and colonic epithelial cells, which further led to increased CXCL9 levels in plasma and cerebrospinal fluid (CSF). CXCL9 induced endoplasmic reticulum (ER) stress in hippocampal neurons by activating the CXCR3-HSPA5 axis, thus leading to depression. Our findings highlight perforin and CXCL9 as possible therapeutic targets for ameliorating mental health sequelae in IBD.

Materials and Methods

Human Tissue and Cells and Clinical Information

This study was approved by the ethics committees of Beijing Friendship Hospital, Capital Medical University (2022-P2-428-01). Healthy volunteers and patients with UC were recruited from Beijing Friendship Hospital between February 2023 and October 2024. All subjects provided written informed consent to participate in the study. Adult outpatients and inpatients aged 18 to 75 years were potentially eligible for inclusion. Those who were diagnosed with IBD according to the "Consensus Opinions on Diagnosis and Treatment of Inflammatory Bowel Disease (2018·Beijing)" standards[9] and who provided clinical information relevant to IBD, completed questionnaires, and provided specimens were included in the study. The exclusion criteria included the following: an unclear diagnosis of IBD; use of immunosuppressive drugs within the past year; presence of abdominal organ tumors in the past 3 years; serious primary diseases of the liver, kidney, or hematopoietic system; or poor control of diabetes, hepatitis B, AIDS, or rheumatic immune-related diseases. All subjects completed a questionnaire under the guidance of a professional physician. The content of the questionnaire included the patient's age, sex, IBD-related information, and questions from the 16-Item Quick Inventory of Depressive Symptomatology (QIDS)[10] and Simple Clinical Colitis Activity Index (SCCAI).[11] All information regarding patients with UC was categorized and presented in Supplementary Table 1, according to the patients included in each experiment. The information included gender, age, body mass index, surgical history, initial diagnosis date, treatment methods, and disease activity. After providing consent, we collected the patients' peripheral blood (approximately 4 mL). We obtained a biopsy sample of intestinal tissue (approximately 3 × 3 × 3 mm) from the subjects who underwent endoscopy. In addition, we enrolled 70 people who had not previously been diagnosed with an underlying disease as a healthy control group.

Mice

Male C57BL/6J mice, B6.*Rag1*-knockout (*Rag1*$^{-/-}$), and B6.*Prf1*-knockout (*Prf1*$^{-/-}$) mice were purchased from The Jackson Laboratory. Male *Prf1/IRES-EGFP-T2A-Cre* (*Prf1*-EGFP) mice were generated on the C57BL/6J background by the Shanghai Model Organisms Center as previously described.[12] The mice were 6 to 8 weeks of age at the beginning of the experiments and maintained in a specific pathogen-free, temperature-controlled environment on a 12-hour light/dark cycle at Beijing Friendship Hospital. All the data derived from the animal studies were analyzed by an experimenter who was blinded to the experimental conditions. The order in which the animals performed the behavioral tests was also randomized. All animal experiments and experimental protocols were approved by the Institutional Animal Care and Ethics Committee of Beijing Friendship Hospital.

Dextran Sulfate Sodium–Induced Colitis Model

Mice aged 6 to 8 weeks were administered a 2.5% dextran sulfate sodium (DSS, wt/vol, 36–50 kDa, MP Biomedicals) solution via drinking water for 7 days.[13] The DSS solution–containing water was refreshed every 3 days. The mice were then given normal drinking water for an additional 2 days. During the recovery phase, behavioral tests were conducted on the mice. The mice were weighed during the colitis modeling process and euthanized at the indicated time points. Colon tissues were collected for further analyses. The disease activity index and histological scores were calculated as previously described[14] (Supplementary Table 2).

$CD8^+$ T-Cell Transfer-Induced Colitis Model

The spleens of C57BL/6J mice and *Prf1*$^{-/-}$ mice were removed and placed individually in ice-cold RPMI 1640.

Following removal, the organs were homogenized, and the resulting suspensions were filtered through a 70-μm cell filter. The filtrates were collected and then centrifuged at 500g for 5 min. Erythrocyte lysis buffer (Qiagen, 79217) was added to the cell pellets, and the cells were lysed on ice for 3 minutes. The lysis process was halted by adding phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). T lymphocytes were subsequently enriched using the Mouse $CD3^+$ T-cell Enrichment Column Kit (R&D, MTCC-25). The samples were incubated with an anti-phycoerythrin antibody (anti-mouse TER119/CD25/CD19/CD4/CD44/NK1.1/CD11b/TCR$\gamma\delta$) at 4°C for 15 minutes in the dark and then with anti-phycoerythrin MicroBeads (Miltenyi, 130-048-801) at 4°C for 15 minutes. Negative selection was performed using LS Columns (Miltenyi, 130-042-401), resulting in the isolation of naïve $CD8^+$ T cells.

A total of 1×10^6 naïve $CD8^+$ T cells from mice (purity $>$ 90%) were adoptively transferred into *B6.Rag1*$^{-/-}$ mice via tail vein injection, and body weight changes were detected over a 6-week period.

Isolation of Colonic Cells

Colons were removed, opened longitudinally, and rinsed with ice-cold PBS. The dissected intestinal tissues were cut into approximately 0.5-cm pieces, and intestinal epithelial cells were dissociated by incubation in Hank's balanced salt solution (Sigma-Aldrich) containing 10 mM EDTA (Thermo Fisher Scientific), 1 mM DTT (Sigma-Aldrich), and 4% FBS with shaking at 130 rpm for 20 minutes at 37°C. The samples were rinsed with PBS after epithelial cell dissociation. The remaining tissues were enzymatically digested in RPMI containing 10 mM HEPES (Gibco), 0.5 mg/mL collagenase D (Sigma-Aldrich), 0.5 mg/mL DNase I (Sigma-Aldrich), and 4% FBS on a shaker at 90 rpm for 40 minutes at 37°C. The digested tissues were passed through a 70-μm cell filter and centrifuged at 500g for 5 minutes. Leukocytes were enriched by 40% Percoll gradient centrifugation and resuspended in PBS containing 2% FBS, followed by antibody incubation.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software). Animal exclusion based on the identification of outliers (ROUT method) using GraphPad Prism 9.0 software was not made for data analyses. To determine the appropriate statistical tests, we initially assessed whether the data followed a Gaussian distribution. Parametric statistical tests were deemed appropriate and subsequently used when the data exhibited both a normal distribution and similar variances between the groups being compared. In cases in which the data did not conform to a normal distribution, nonparametric statistical tests were applied. Statistical significance was determined using 2-tailed Student t tests (unpaired) for comparisons between 2 groups. For multiple group comparisons, 1-way analysis of variance with Tukey's multiple comparison test was used. For multiple comparisons, 2-way analysis of variance with Sidak's multiple comparison test was used. All data are presented as the mean ± standard error of the mean. Significance levels are indicated as follows: *$.01 < P < .05$, **$.001 < P < .01$, ***$P < .001$; "ns" denotes no statistical significance.

For further details regarding the materials and methods used, please refer to the Supplementary Materials.

Results

Perforin Expressed by $CD8^+$ T Cells Was Associated With IBD-Induced Depression in Both Patients and Mouse Models

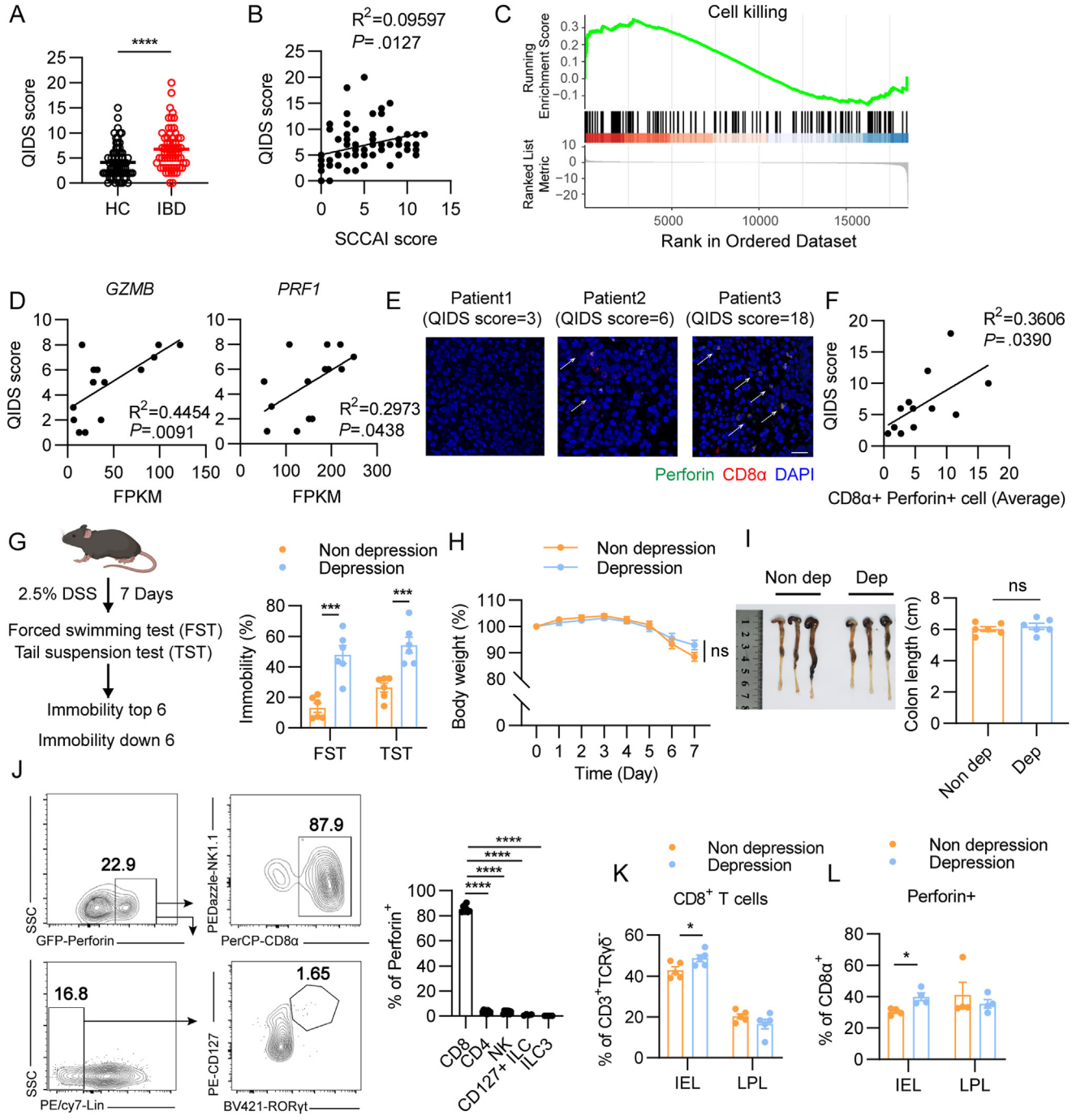
To investigate the relationship between depressive symptoms and bowel inflammation in humans, we analyzed responses of 64 patients with UC and 70 healthy controls to the QIDS and SCCAI questionnaires in this observational study. The demographic and clinical characteristics of the patients and healthy controls are shown in Supplementary Table 3. Patients with UC exhibited an increased risk of depression, as reflected by higher QIDS scores (Figure 1*A*). Interestingly, the depressive symptoms (QIDS scores) were positively correlated with the progression of enteritis (SCCAI scores) in patients with UC (Figure 1*B*). Because predominant intestinal inflammatory cell infiltration is observed in patients with IBD, we speculated that the induction of depression resulted from changes in intestinal tissue. We then characterized colonic changes by whole-transcriptome RNA sequencing (RNA-seq). Gene set enrichment analysis revealed enrichment of genes associated with the cell-killing pathway in patients with UC with depression (Figure 1*C*). The levels of *PRF1* and *GZMB*, among various effector molecules, were positively correlated with the QIDS scores, whereas the levels of other granzymes or cytokines, such as *GZMA* and *IL17A*, were not significantly correlated with these scores (Figure 1*D*, Supplementary Figure 1*A*). Perforin, encoded by the *PRF1* gene, is the pivotal rate-limiting molecule for the cytotoxic function of lymphocytes and is required for delivering granzymes into target cells. We analyzed the source of perforin using immunofluorescence staining, which revealed that it was mainly produced by $CD8^+$ T cells rather than natural killer cells (Figure 1*E*, Supplementary Figure 1*B*). Moreover, the infiltration of $CD8^+$perforin$^+$ T cells increased in the colonic epithelium of patients with UC with depression (Figure 1*E* and *F*).

We further generated a mouse model of colitis using DSS and evaluated depressive-like behaviors in the mice after DSS treatment. The forced swimming test and tail suspension test are classical acute stress assays used to measure immobile time, which is positively correlated with the level of depression. We ranked the colitis model mice according to their immobility time in the forced swimming test and tail suspension test, assigning the 6 mice that exhibited the longest immobility time to the "depression" group and those with the shortest immobility time to the "non depression" group (Figure 1G). There was no significant difference in body weight, colon length and weight, spleen weight, and colitis severity between the 2 groups (Figure 1*H* and *I*, Supplementary Figure 2*A–D*). Moreover, we observed comparable levels in anxiety-like behaviors and motor ability between the 2 groups of mice (Supplementary Figure 2*E–G*). We subsequently determined that $CD8^+$ T cells were the main source of perforin in the colon

(Figure 1*J*). We assessed the composition of the intestinal immune cell population in colitis mice with or without depression by flow cytometric analysis and found a significant increase in the proportion of $CD8^+$ T cells among intraepithelial lymphocytes but not among lamina propria lymphocytes and a significant increase in perforin production by $CD8^+$ T cells in depressed mice (Figure 1*K* and *L*, Supplementary Figure 3*A* and *B*). These results suggested that perforin in $CD8^+$ T cells may be associated with IBD-induced depression.

Perforin Deficiency in $CD8^+$ T Cells Relieved Colitis-Induced Depression

To determine whether a high level of perforin participates in IBD-driven depression, wild-type (WT) or *Prf1*$^{-/-}$ mice were randomly delegated to exposure to normal water or DSS for 7 days. Deletion of *Prf1* did not result in any significant alterations in body weight, colon and spleen weight, or anxiety and depressive-like behaviors under regular water (Supplementary Figure 4*A*–*H*). After DSS administration, *Prf1*$^{-/-}$ mice exhibited markedly decreased



inflammation severity, including decreased body weight loss and reduced rectal bleeding and diarrhea (Figure 2*A* and *B*). Histologically, *Prf1*$^{-/-}$ mice treated with DSS exhibited a marked increase in colon length, decrease in colon weight, and alleviation of intestinal epithelial damage, without significant change of spleen weight (Figure 2*C–F*, Supplementary Figure 5*A–C*). Moreover, *Prf1*$^{-/-}$ mice exhibited less severe despair rather than anxiety-like behaviors, as indicated by a shorter immobility time in the forced swimming test and tail suspension test than did their WT counterparts (Figure 2*G* and *H*, Supplementary Figure 5*D–F*).

We then directly tested the hypothesis that CD8$^+$ T-cell–derived perforin inhibits inflammation and depression using a CD8$^+$ T-cell transfer-induced colitis model. As shown in Figure 2*I*, naïve CD8$^+$ T cells (CD44^{low}CD62L$^+$CD8a$^+$ cells) were isolated from the spleens of WT or *Prf1*$^{-/-}$ mice and intravenously injected into lymphopenic recipient *Rag1*$^{-/-}$ mice. Adoptive transfer of *Prf1*$^{-/-}$ naïve CD8$^+$ T cells showed mitigated colitis, as evidenced by reduced weight loss in recipient *Rag1*$^{-/-}$ mice through weeks 4 to 6 after CD8$^+$ T-cell transfer (Figure 2*J*). Deficiency of perforin in CD8$^+$ T cells caused remarkable improvement of colon length (Figure 2*K* and *L*), reduction in the severity of pathology, including thinning of the large intestinal wall, and reduced lamina propria inflammatory infiltration (Figure 2*M* and *N*). *Rag1*$^{-/-}$ mice receiving *Prf1*$^{-/-}$ CD8$^+$ T cells exhibited less severe despair-related behaviors in the forced swimming test and tail suspension test (Figure 2*O*). Taken together, these findings established a central role for perforin in regulating CD8$^+$ T cells to exacerbate colitis-induced depression and identified perforin as an attractive target for treating IBD-driven depressive-like behaviors.

Perforin Exacerbated Colitis-Induced Depression by Upregulating the Expression of CXCL9 in Intestinal Epithelial Cells

The mammalian gut harbors a complex ecosystem that is critical for maintaining gut homeostasis.[15] Intestinal epithelial cells constitute the first physical and immunological protective barrier.[16] To investigate the pivotal genes in the colon whose expression is affected by perforin during depression induced by colitis, we analyzed the transcriptome of colonic tissue from depressed or *Prf1*-deficient mice. A total of 3 overlapping differentially expressed genes were identified between the DSS-treated mice in the depression group and nondepression group and between the DSS-treated WT mice and *Prf1*$^{-/-}$ mice (Figure 3*A*). Among the differentially expressed genes, *Cxcl9* is the only chemokine gene that has recently been shown to be highly predictive of UC.[17] At the protein level, we also verified an increase in the concentration of CXCL9 in the plasma of depressed mice and patients with UC with severe depressive symptoms (Figure 3*B* and *C*). We subsequently demonstrated the role of CXCL9 in colitis-related depression using CXCL9-neutralizing antibodies. Neutralizing CXCL9 alleviated depression but had no effect on colitis in mice (Figure 3*D–G*). Immunofluorescence assays indicated that EpCAM$^+$ intestinal epithelial cells were the dominant producers of CXCL9. Compared with that in the colonic epithelial cells of WT mice, the expression of CXCL9 in the colonic epithelial cells of *Prf1*$^{-/-}$ mice was significantly downregulated (Figure 3*H–J*, Supplementary Figure 6*A*). Furthermore, the protein level of CXCL9 was significantly decreased in the plasma, CSF, and hippocampus of *Prf1*$^{-/-}$ mice (Figure 3*K*).

CD8$^+$ T-Cell–Derived Perforin and GzmB Promoted the Expression of CXCL9 in Colonic Epithelial Cells

Because CXCL9 is generally considered to be stimulated by interferon (IFN)-γ,[18] we first investigated the proportion of lymphocytes in WT and *Prf1*$^{-/-}$ colitis mice and IFN-γ production by these cells. Perforin deficiency did not impact the proportion of CD8$^+$ T cells in the colon (Supplementary Figure 7*A*) or the expression of IFN-γ by CD4$^+$ and CD8$^+$ T cells in the colon (Supplementary Figure 7*B*). Moreover, the expression of another cytotoxic molecule that synergizes

Figure 1. Perforin expressed by CD8$^+$ T cells was associated with IBD-induced depression in both patients and mouse models. (*A*) The 16-Item QIDS to assess depressive severity in healthy controls (n = 70) and patients with UC (n = 64), unpaired *t* test with Welch's correction, $P < .0001$. (*B*) Correlation analysis of SCCAI and QIDS score in patients with UC (n = 64). (*C*) Gene set enrichment analysis on patients with UC with nondepression vs depression expression data of colonic epithelial samples. (*D*) Correlation analysis of colonic *PRF1* and *GZMB* expression and QIDS in patients with UC (n = 14), 2 repeat experiments. (*E*) Immunofluorescent staining with CD8α and perforin in the colon of patients with UC. *Scale bar* = 20 μm. (*F*) Correlation analysis of colonic CD8$^+$Perforin$^+$ T cells and QIDS in patients with UC (n = 12), 4 repeat experiments. (*G*) *Left*: schematic of the experimental design of screening mice with colitis-induced depression; *Right*: the percentage of immobility time compared between colitis mice with depression (n = 6) or not (n = 6) in forced swimming test (FST) and tail suspension test (TST), unpaired *t* test, $P_{FST} = .0003$, $P_{TST} = .0009$, 2 repeat experiments. (*H*) Body weight of nondepression (n = 8) and depression (n = 8) mice with colitis given 2.5% DSS in drinking water for 7 days, 2-way analysis of variance (ANOVA) with Sidak's multiple comparisons test, $P = .7251$, 2 repeat experiments. (*I*) Representative images and statistical analysis of colon length from nondepression (n = 6) and depression (n = 6) mice with colitis, unpaired *t* test, $P = .5109$, 2 repeat experiments. (*J*) Flow cytometry image and statistical analysis of perforin$^+$ cells relative to the total numbers of CD8$^+$, NK1.1$^+$, CD4$^+$, CD127$^+$ ILC, and RORγt$^+$CD127$^+$ ILC3 cells in the colon of *Prf1*-EGFP mice, n = 4–7, 1-way ANOVA with Tukey's multiple comparisons test, $P < .0001$, 3 repeat experiments. (*K*) CD45$^+$CD3$^+$TCRγδ$^-$CD8α$^+$CD8β$^+$ cells in intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) obtained from colitis mice with depression (n = 5) or not (n = 5) were quantified by flow cytometric analysis, unpaired *t* test, $P_{IEL} = .0399$, $P_{LPL} = .1910$, 2 repeat experiments. (*L*) CD45$^+$CD3$^+$TCRγδ$^-$CD8α$^+$perforin$^+$ cells in IEL and LPL obtained from colitis *Prf1*-EGFP mice with depression (n = 4) or not (n = 4) were quantified by flow cytometric analysis, unpaired *t* test, $P_{IEL} = .0125$, $P_{LPL} = .5299$, 2 repeat experiments. ns, nonsignificant.

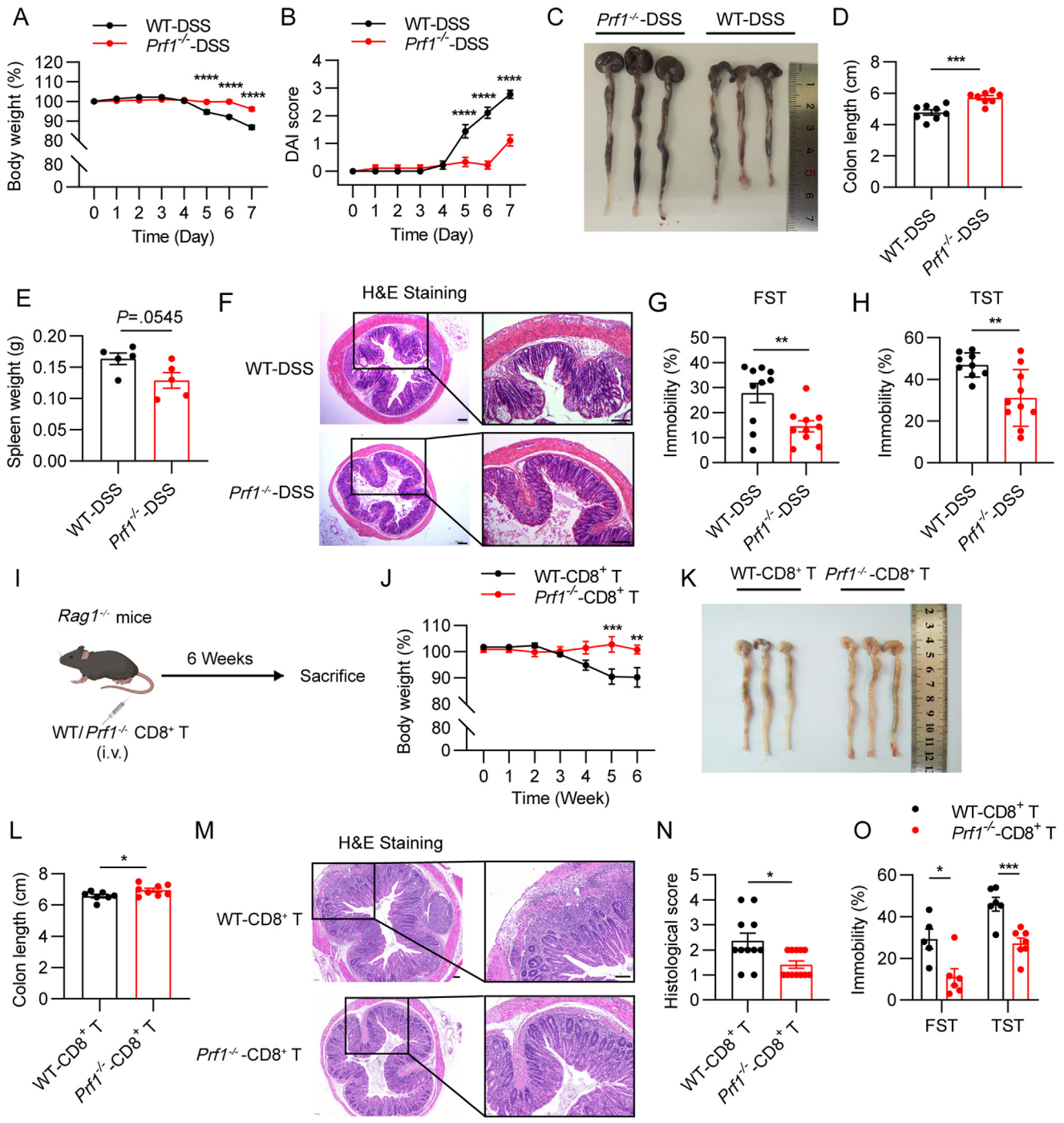


Figure 2. Deficiency of perforin in $CD8^+$ T cells relieved colitis-induced depression. (*A*) Body weight and (*B*) disease scores of WT (n = 9) and $Prf1^{-/-}$ (n = 9) mice given 2.5% DSS in drinking water for 7 days, 2-way analysis of variance (ANOVA) with Sidak's multiple comparisons test, $P < .0001$, 3 repeat experiments. (*C*) Representative image of colon length from WT and $Prf1^{-/-}$ mice. (*D* and *E*) Statistical analysis of (*D*) colon length and (*E*) spleen weight from WT (n = 8) and $Prf1^{-/-}$ (n = 8) mice, unpaired *t* test, $P_{\text{Colon length}} = .0006$, $P_{\text{Spleen weight}} = .0545$, 3 repeat experiments. (*F*) Distal section of the colons examined by hematoxylin-eosin (H&E) staining. *Scale bar* = 100 μm. (*G* and *H*) The percentage of immobility time compared between WT-DSS (n = 10) and $Prf1^{-/-}$-DSS (n = 10) mice in (*G*) forced swimming test (FST), unpaired *t* test, $P = .0078$, and (*H*) tail suspension test (TST), unpaired *t* test with Welch's correction, $P = .0053$, 2 repeat experiments. (*I*) Schematic illustration and timeline of adoptive transfer colitis model. (*J*) Body weight of recipient $Rag1^{-/-}$ mice receiving WT (n = 6) or $Prf1^{-/-}$ (n = 7) naïve $CD8^+$ T cells, 2-way ANOVA with Sidak's multiple comparisons test, $P = .0005$, 2 repeat experiments. (*K*) Representative images and (*L*) statistical analysis of colon length from recipient $Rag1^{-/-}$ mice, unpaired *t* test, $P = .0450$, 2 repeat experiments. (*M*) Representative H&E sections of colons of recipient $Rag1^{-/-}$ mice. *Scale bar* = 100 μm. (*N*) Quantification of H&E staining as measured by the histological score, Mann-Whitney test, $P = .0193$, 3 repeat experiments. (*O*) The percentage of immobility time compared between recipient $Rag1^{-/-}$ mice receiving WT (n = 6) or $Prf1^{-/-}$ (n = 7) naïve $CD8^+$ T cells in forced swimming test (FST) and tail suspension test (TST), unpaired *t* test, $P_{\text{FST}} = .0158$, $P_{\text{TST}} = .0009$, 2 repeat experiments.

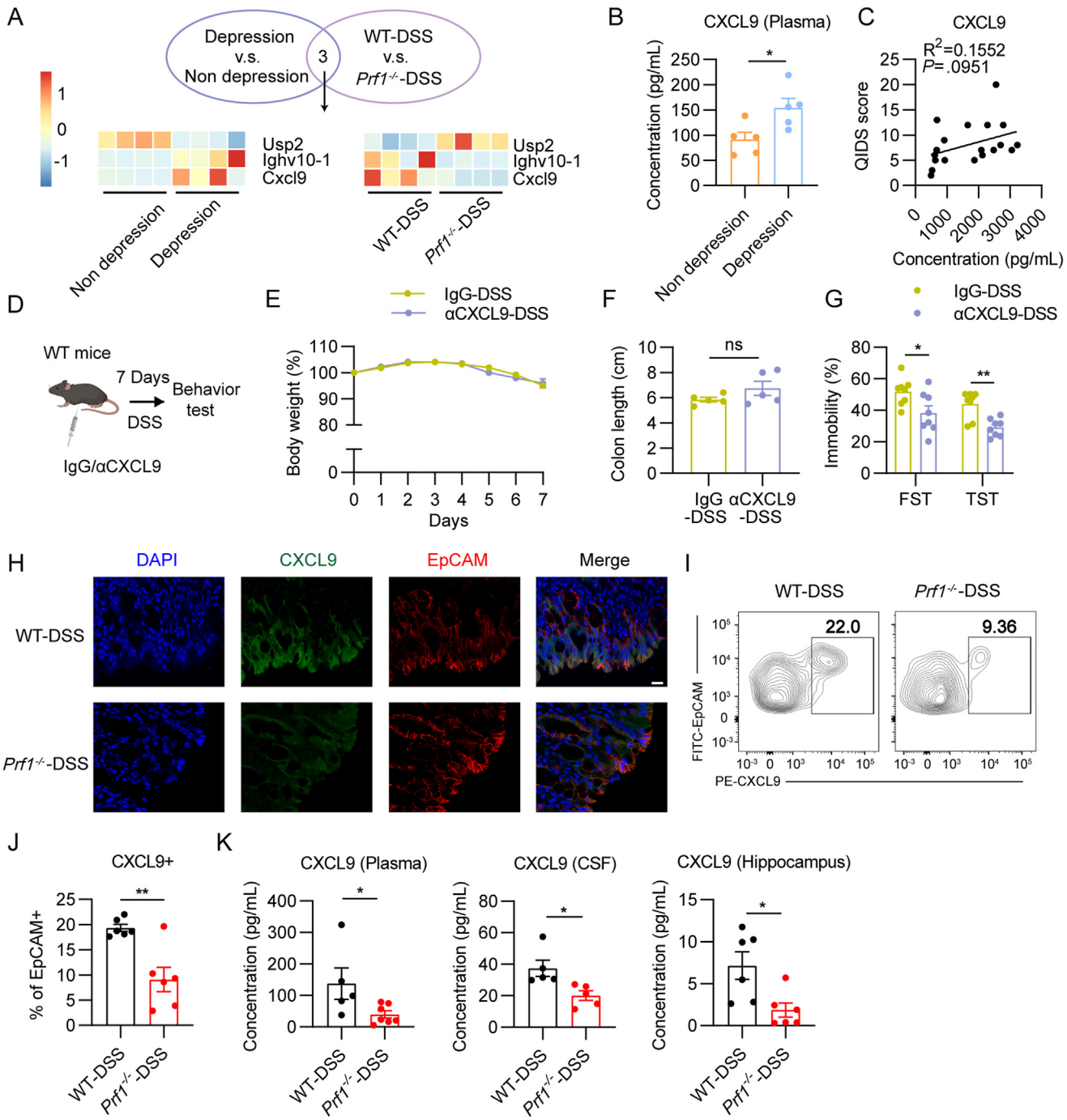


Figure 3. Perforin exacerbated colitis-induced depression by upregulating the level of CXCL9 in intestinal epithelial cells. (*A*) Genes were obtained from the intersection of depression-DSS vs nondepression-DSS mice and WT-DSS vs *Prf1*$^{-/-}$-DSS mice. (*B*) CXCL9 concentration in the plasma of colitis mice with depression (n = 5) or not (n = 5), unpaired *t* test, $P = .0273$, 2 repeat experiments. (*C*) Correlation analysis of CXCL9 concentration in plasma and QIDS in patients with UC (n = 19), 2 repeat experiments. (*D*) Schematic illustration and timeline of intraperitoneal injection of CXCL9 neutralizing antibody in WT mice. (*E*) Body weight of WT mice receiving immunoglobulin (Ig)G (n = 8) or CXCL9 neutralizing antibody (n = 8) followed by DSS treatment for 7 days, 2-way analysis of variance with Sidak's multiple comparisons test, $P = .6658$, 2 repeat experiments. (*F*) Statistical analysis of colon length from WT mice receiving IgG (n = 5) or CXCL9 neutralizing antibody (n = 5), unpaired *t* test with Welch's correction, $P = .1932$. (*G*) The percentage of immobility time compared between WT mice receiving IgG (n = 8) and CXCL9 neutralizing antibody (n = 8) in forced swimming test (FST) and tail suspension test (TST), unpaired *t* test, $P_{FST} = .0258$, $P_{TST} = .0010$, two repeat experiments. (*H*) Immunofluorescent staining for CXCL9 and EpCAM in colon tissue of WT and *Prf1*$^{-/-}$ mice with colitis. *Scale bar* = 20 μm. (*I*) Flow cytometry image and (*J*) statistical analysis of CXCL9^{+} cells relative to the total numbers of EpCAM^{+} epithelial cells in the colon of WT (n = 6) and *Prf1*$^{-/-}$ (n = 6) mice with colitis, unpaired *t* test with Welch's correction, $P = .0073$, 2 repeat experiments. (*K*) CXCL9 concentration in the plasma, CSF, and hippocampus of WT and *Prf1*$^{-/-}$ mice with colitis, unpaired *t* test, $P_{Plasma} = .0473$, $P_{CSF} = .0205$, $P_{Hippocampus} = .0167$, 3 repeat experiments.

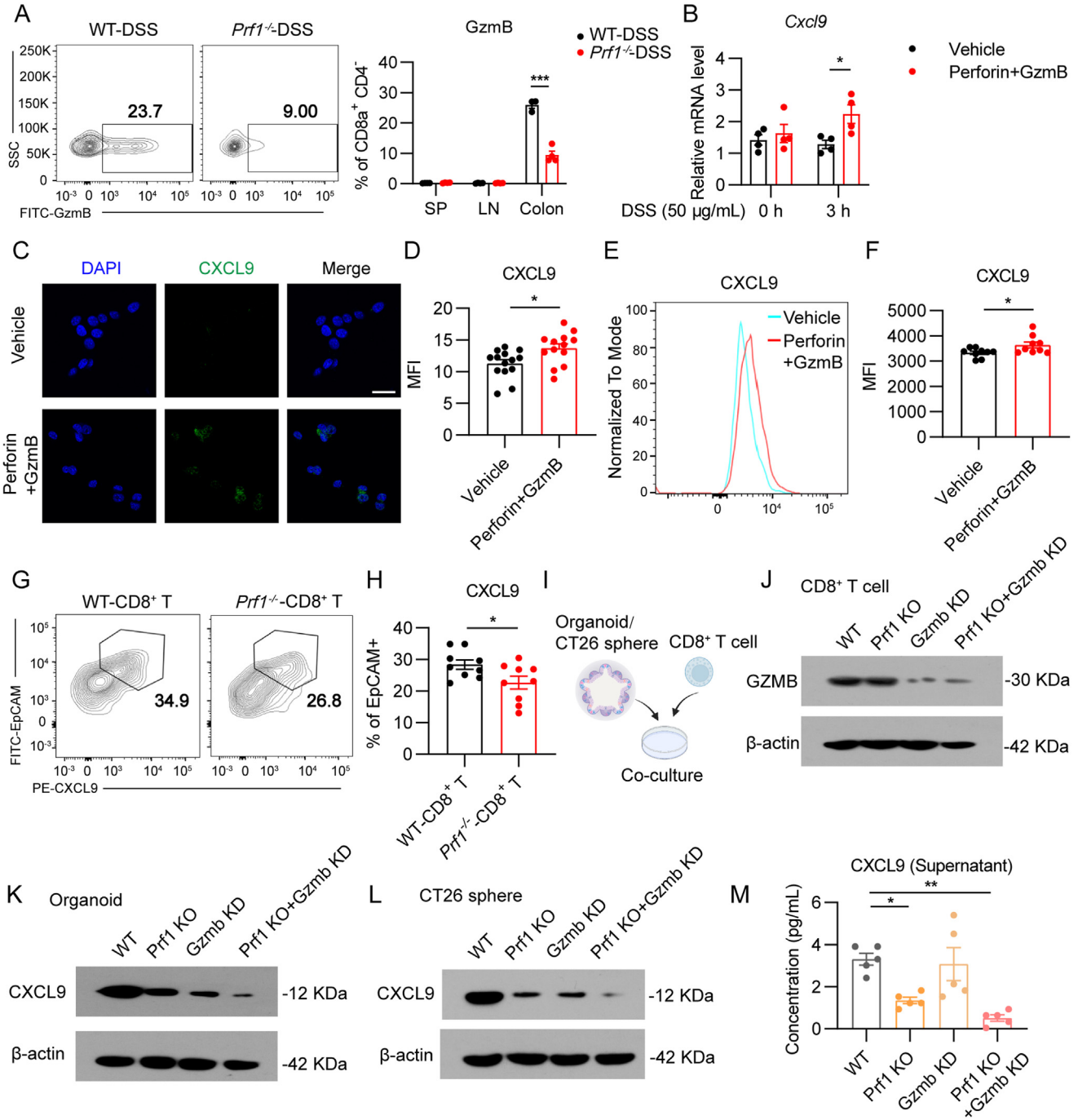


Figure 4. CD8$^+$ T-cell–derived perforin and GzmB promoted the expression of CXCL9 in colonic epithelial cells. (*A*) Flow cytometry image and statistical analysis of GzmB$^+$ cells relative to the total numbers of CD8$^+$ T cells in the mLN, spleen, and colon of WT (n = 4) and *Prf1*$^{-/-}$ (n = 4) mice with colitis, unpaired *t* test, $P_{SP} = .1042$, $P_{LN} = .5097$, $P_{Colon} = .0003$, 2 repeat experiments. mLN, mesenteric lymph node. (*B*) Mouse colon epithelial cell line CT26 was stimulated with DSS (50 μg/mL) with addition of vehicle or perforin (1 μg/mL) and GzmB (1 μg/mL) at indicated time points. The expression of *Cxcl9* messenger RNA (mRNA) was examined by quantitative real-time PCR (qPCR), 2-way analysis of variance (ANOVA) with Sidak's multiple comparisons test, $P_{0h} = .7930$, $P_{3h} = .0257$, 2 repeat experiments. (*C* and *D*) Immunofluorescent staining and (*E* and *F*) flow cytometry analysis of the CXCL9 expression in CT26 stimulated with vehicle or perforin and GzmB at 3 hours, unpaired *t* test, $P_{IF} = .0141$, $P_{FACS} = .0364$, 2 repeat experiments. *Scale bar* = 20 μm. (*G*) Flow cytometry image and (*H*) statistical analysis of CXCL9$^+$ cells relative to the total numbers of EpCAM$^+$ epithelial cells in the colon of recipient *Rag1*$^{-/-}$ mice receiving WT (n = 9) or *Prf1*$^{-/-}$ (n = 9) naïve CD8$^+$ T cells, unpaired *t* test, $P = .0352$, 2 repeat experiments. (*I*) Schematic illustration of co-culture of organoids or CT26 spheres with CD8$^+$ T cells. (*J*) GzmB expression in WT or *Prf1*$^{-/-}$ (*Prf1* KO) CD8$^+$ T cells transfected with Gzmb siRNA (*Gzmb* KD) or control siRNA measured by Western blot. (*K*) CXCL9 expression in organoids co-cultured with WT, *Prf1* KO, *Gzmb* KD, or *Prf1* KO + *Gzmb* KD CD8$^+$ T cells measured by Western blot. (*L*) CXCL9 expression in CT26 spheres co-cultured with WT, *Prf1* KO, *Gzmb* KD, or *Prf1*/*Gzmb* DKO CD8$^+$ T cells measured by Western blot. (*M*) CXCL9 expression in supernatant of organoids co-cultured with WT, *Prf1* KO, *Gzmb* KD, or *Prf1*/*Gzmb* DKO CD8$^+$ T cells measured by enzyme-linked immunosorbent assay (ELISA), n = 5, 1-way ANOVA with Tukey's multiple comparisons test, $P_{Prf1KO} = .0244$, $P_{GzmbKD} = .9790$, $P_{Prf1KO+GzmbKD} = .0015$.

with perforin, GzmB, was significantly downregulated in colonic $CD8^+$ T cells from *Prf1*$^{-/-}$ mice (Figure 4*A*, Supplementary Figure 6*B*). To verify the direct effect of perforin and GzmB on intestinal epithelial cells, we simulated colitis by stimulating CT26 colonic cells with DSS solution in vitro. After stimulation with perforin and GzmB for 3 hours, CT26 significantly upregulated CXCL9 at both the messenger RNA and protein levels (Figure 4*B*–*F*). However, the expression of CXCL9 was not increased by perforin stimulation alone (Supplementary Figure 8*A* and *B*). To determine whether $CD8^+$ T-cell–derived perforin and GzmB upregulated CXCL9 expression in intestinal epithelial cells, we used a $CD8^+$ T-cell transfer-induced colitis model. We found that CXCL9 expression was decreased in colonic epithelial cells in *Rag1*$^{-/-}$ mice receiving *Prf1*$^{-/-}$ $CD8^+$ T cells (Figure 4*G* and *H*). To investigate the synergistic effect of GzmB and perforin, we performed co-cultures of intestinal organoids and mucosal $CD8^+$ T cells isolated from WT or *Prf1*$^{-/-}$ (*Prf1* KO) mice transfected with *Gzmb* small interfering RNA (siRNA) (*Gzmb* KD) or control siRNA (Figure 4*I*). As expected, $CD8^+$ T cells that received *Gzmb* siRNA delivery had decreased levels of GzmB compared with those receiving control siRNA (Figure 4*J*). Using this approach, we next examined the consequences of *Gzmb* knockdown under conditions of co-cultures. Deletion of either *Prf1* or *Gzmb* in $CD8^+$ T cells led to a decrease in CXCL9 expression in co-cultured organoids and CT26 spheres, whereas downregulation of CXCL9 expression was amplified in the simultaneous absence of both *Prf1* and *Gzmb* (Figure 4*K*–*M*, Supplementary Figure 9*A*–*C*). These results indicated that $CD8^+$ T-cell–derived perforin and GzmB promoted the expression of CXCL9 in colonic epithelial cells.

CXCL9 Contributed to ER Stress in Hippocampal Neurons Via the CXCR3-HSPA5 Axis

We sought to understand the underlying mechanism by which perforin and CXCL9 regulate depressive-like behaviors in mice with colitis. Because the hippocampus and medial prefrontal cortex are the main brain regions regulating emotion and the progression of depressive-like behaviors, we investigated transcriptome alterations in these regions in both depressed and nondepressed DSS-treated mice. Four differentially expressed genes were significantly altered in both regions simultaneously (Figure 5*A*, Supplementary Figure 10*A* and *B*). Interestingly, among these 4 genes, *Hspa5* was shown to be downstream of the CXCL9 receptor CXCR3.[19] In addition, *Hspa5* encodes the ER stress-related protein BiP, which is located in the ER, senses unfolded proteins, and initiates ER stress.[20] GSVA scores of ER stress pathway in the hippocampus of depressed WT mice with DSS treatment were higher than nondepressed individuals (Supplementary Figure 10*C*). Moreover, we confirmed the downregulation of *Hspa5* in the hippocampal neurons of DSS-treated *Prf1*$^{-/-}$ mice (Figure 5*B* and *C*). A decrease in the level of the apoptotic marker cleaved caspase 3 in neurons was also observed in the hippocampus of DSS-treated *Prf1*$^{-/-}$ mice (Figure 5*C*). To verify whether the CXCL9 protein mediates *Hspa5* expression in neurons and depressive-like behaviors, we intracerebroventricularly injected CXCL9 into DSS-treated *Prf1*$^{-/-}$ mice (Figure 5*D*). The severity of colitis in *Prf1*$^{-/-}$ mice that received CXCL9 was similar to that in mice that received the vehicle (Supplementary Figure 11*A* and *B*). CXCL9 significantly increased the severity of depressive-like behaviors and the expression of *Hspa5* in DSS-treated *Prf1*$^{-/-}$ mice (Figure 5*E*–*G*). Consequently, the expression of other classical ER stress genes, *Atf4*, *Erp5,* and *Xbp1,* was significantly increased in the hippocampus of *Prf1*$^{-/-}$ mice after CXCL9 injection (Figure 5*H*). To further verify that IBD-induced CXCL9 circulated throughout the body to the hippocampus where it can affect neurons, we added plasma from WT or DSS-treated WT mice to the neuronal culture system (Figure 5*I*). CXCL9 levels in the plasma of control mice were remarkably lower than those in the plasma of DSS-treated mice (Figure 5*J*). The WT-DSS mouse plasma induced significantly higher levels of *Hspa5* expression in neurons than that of WT mouse plasma, which can be notably reversed by neutralizing antibodies against CXCL9 (Figure 5*K* and *L*). Therefore, we demonstrated that circulating CXCL9 contributed to ER stress in hippocampal neurons.

Discussion

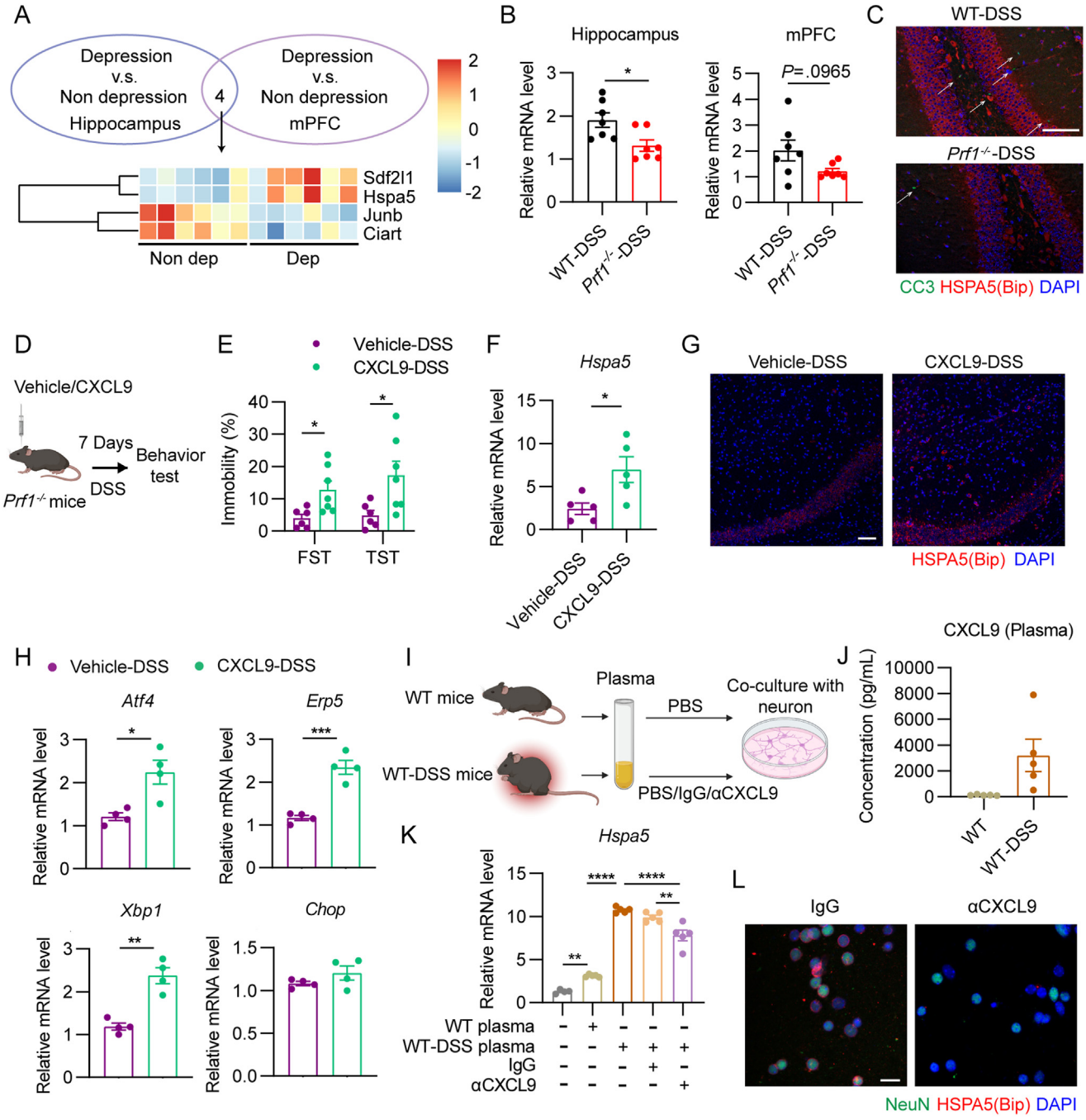
Patients with IBD exhibited an increased risk of depression, but the mechanisms underlying this association remain unclear. Previous studies have primarily focused on the alterations in gut immune cells and the levels of brain inflammation associated with IBD-induced depression. An imbalanced T helper type-17 (Th17)/T regulatory (Treg) cell ratio and an increase in peripheral $\alpha4\beta7^+$ monocyte populations can exacerbate mood disorders associated with IBD.[21,22] The alterations in peripheral immune cells and the rise in proinflammatory cytokines contribute to increased blood-brain barrier permeability, leading to elevated levels of proinflammatory cytokines in emotion-regulating brain regions, such as the hippocampus.[23,24] This cascade results in the overactivation of astrocytes and microglia, ultimately culminating in depression.[25]

Our study also identified a cascade of cellular and molecular events that link the exacerbation of enteric inflammation to depression (Figure 6). We observed a significantly greater percentage of perforin$^+$$CD8^+$ T cells infiltrating the colons in patients with UC and colitis model mice with depression. In the colon, $CD8^+$ T cells are the primary source of perforin. Perforin deficiency in $CD8^+$ T cells resulted in reduced susceptibility to colitis and alleviation of multiple depressive-like behaviors. The in vitro and in vivo experiments indicated that perforin produced by $CD8^+$ T cells along with GzmB to promote CXCL9 expression in intestinal epithelial cells. Moreover, the CXCL9-CXCR3-HSPA5 axis was found to possibly contribute to the induction of depressive-like behaviors in DSS-treated mice, which could be reversed in *Prf1*$^{-/-}$ mice.

From an evolutionary perspective, human vulnerability to depression promotes host survival and reproduction in highly pathogenic environments.[26,27] Depressive symptoms

have been observed among patients with distinct immune-mediated inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, and ovarian cancer.[4,28,29] Interestingly, the expansion of $CD8^+$ T cells was observed in depressed individuals with these diseases.[29–31] Herein, we found that the proportion of perforin$^+$$CD8^+$ T cells was increased in the colons of patients with UC and colitis model mice with depression.

Currently, the unbalanced Th17/Treg cell ratio has been widely considered the main culprit behind IBD-induced depression.[32] However, the potential contribution of $CD8^+$ T cells to IBD-induced depression remains largely unexplored. We found that in patients with IBD with depression, severe colitis was associated with increased infiltration of $CD8^+$ T lymphocytes into the colonic mucosa. The expression of perforin, a key effector molecule, was also upregulated in $CD8^+$ T cells from patients with IBD and colitis model mice with depression, whereas IFN-γ and interleukin-17 levels remained unchanged. In the colon, perforin was expressed primarily by $CD8^+$ T cells, thus



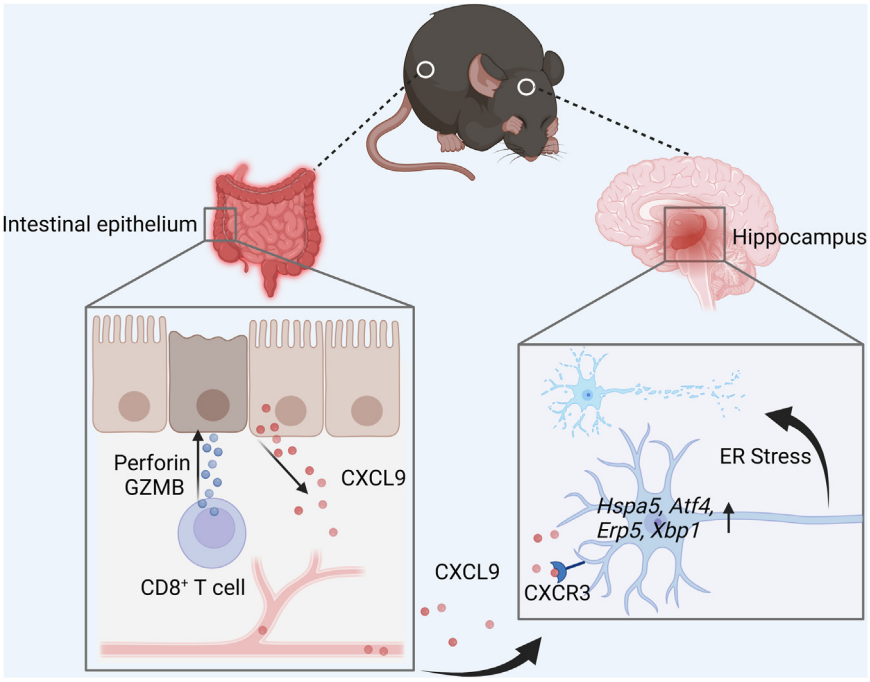


Figure 6. Intrinsic mechanisms of perforin generated by $CD8^+$ T cells regulation on IBD-related depression (created with BioRender.com).

virtually excluding other cells as its source. Perforin deletion inhibited $CD8^+$ T-cell-driven colonic inflammation and the subsequent depressive-like behaviors. These findings underscore the critical role of perforin in IBD-induced depression and further support the link between $CD8^+$ T cells and perforin in contributing to IBD-induced depression.

In this study, we demonstrated that *Prf1* knockout in $CD8^+$ T cells significantly decreased CXCL9 accumulation and secretion in gut epithelial cells. It has been reported that neuroinflammation-induced depression in IBD involves peripheral inflammatory mediators such as tumor necrosis factor-α originating from the inflamed gut penetrating the blood-brain barrier.[33,34] Consistent with the decrease in the concentration of peripheral CXCL9, the concentration of CXCL9 in CSF was also decreased in $Prf1^{-/-}$ colitis model mice. Previous genetic prediction studies and serum proteomic analyses have indicated an association between elevated circulating CXCL9 levels and an increased risk of developing IBD.[35,36] Administration of a CXCL9-specific neutralizing antibody did not affect the severity of colitis; however, it significantly ameliorated depressive-like symptoms. These data suggest that CXCL9 upregulation might be a downstream consequence of colitis and contribute to the development of depression in this context.

Changes in cytokine levels in CSF exert multiple effects by either directly or indirectly activating resident microglia, impairing the maturation and proliferation of hippocampal progenitor cells and promoting neurodegeneration.[37,38] Limited research has investigated how peripheral cytokines

Figure 5. CXCL9 contributed to ER stress in hippocampal neurons via the CXCR3-HSPA5 axis. (*A*) Genes were obtained from the intersection of hippocampus and medial prefrontal cortex (mPFC) in depression-DSS vs nondepression-DSS mice. (*B*) Quantitative real-time polymerase chain reaction (qPCR) detection of *Hspa5* messenger RNA (mRNA) expression levels in the hippocampus and mPFC of WT (n = 7) and $Prf1^{-/-}$ (n = 7) mice subjected to DSS, unpaired *t* test, $P_{Hippocampus} = .0160$; unpaired *t* test with Welch's correction, $P_{mPFC} = .0965$, 2 repeat experiments. (*C*) Immunofluorescence images of HSPA5 (*red*) and cleaved caspase-3 (CC3) (*green*) from WT and $Prf1^{-/-}$ mice treated with 7 days of DSS. *Scale bar* = 20 μm. (*D*) Schematic illustration and timeline of CXCL9 intraventricular injection to $Prf1^{-/-}$ mice. (*E*) The percentage of immobility time compared between $Prf1^{-/-}$ mice receiving vehicle (n = 6) and CXCL9 (n = 7) in forced swimming test (FST) and tail suspension test (TST), unpaired *t* test, $P_{FST} = .0171$; unpaired *t* test with Welch's correction, $P_{TST} = .0269$, 2 repeat experiments. (*F*) qPCR and (*G*) immunofluorescent staining detection of *Hspa5* mRNA and protein expression levels in the hippocampus of $Prf1^{-/-}$ mice receiving vehicle and CXCL9, unpaired *t* test, $P = .0239$, 2 repeat experiments. *Scale bar* = 10 μm. (*H*) qPCR detection of *Atf4*, *Erp5*, *Xbp1*, and *Chop* mRNA expression levels in the hippocampus of $Prf1^{-/-}$ mice receiving vehicle and CXCL9, unpaired *t* test, $P_{Atf4} = .0119$, $P_{Erp5} = .0005$, $P_{Xbp1} = .0011$, $P_{Chop} = .2069$, 2 repeat experiments. (*I*) Schematic illustration of co-culture of primary neurons with the plasma derived from WT or WT-DSS mice supplemented with immunoglobulin (Ig)G or anti-CXCL9 neutralizing antibody. (*J*) CXCL9 expression in the plasma derived from WT (n = 5) or WT-DSS (n = 5) mice measured by enzyme-linked immunosorbent assay (ELISA), unpaired *t* test with Welch's correction, $P = .0727$. (*K*) qPCR and (*L*) immunofluorescent staining detection of *Hspa5* mRNA and protein expression levels in the neurons co-cultured with the plasma supplemented with IgG (n = 5) or anti-CXCL9 neutralizing antibody (n = 5), 1-way analysis of variance with Tukey's multiple comparisons test, $P_{Null\ vs\ WT} = .0094$, $P_{WT\ vs\ WT\text{-}DSS} < .0001$, $P_{IgG\ vs\ \alpha CXCL9} = .0013$.

might influence neural functions involved in behavioral and emotional regulation, potentially leading to the emergence of psychiatric symptoms. This study provides the first evidence that CXCL9 contributes to depressive-like behaviors in mice with colitis by promoting neuronal ER stress. CXCR3, the receptor for CXCL9, is expressed on neurons and microglia and plays an important role in IFN-β-induced depression-like behaviors.[39] CXCR3 induces ER stress by stimulating ER stress regulators, including *Hspa5*.[19] The transcriptome data suggested that *Hspa5* was downregulated in the hippocampus of DSS-treated *Prf1*$^{-/-}$ mice compared with the hippocampus of DSS-treated WT mice. *Prf1*$^{-/-}$ mice that received intracerebroventricular injection of the CXCL9 protein exhibited a significant increase in the severity of depression, accompanied by upregulation of ER stress molecules, including *Hspa5*, *Atf4*, *Erp5*, and *Xbp1*, in the hippocampus. Co-culture experiments indicated that high concentration of CXCL9 in plasma promoted ER stress in neurons, which was reversed by CXCL9 neutralizing antibody. Chronic ER stress augments spontaneous excitatory neurotransmission, which may contribute to abnormalities in neuronal circuitry preceding synapse degeneration.[40] ER stress–regulated activation of the CXCR3 pathway has also been shown to induce neuronal cell death.[41,42] These results indicated that CXCL9, which was upregulated by perforin, worsened colitis-related depression through the activation of neuronal ER stress.

Notably, as a chemokine receptor, CXCR3 is also abundantly expressed on CD8$^+$ T cells. Perforin-mediated upregulation of CXCL9 in epithelial cells could further promote the recruitment of CXCR3-positive CD8$^+$ T cells to the colonic mucosa in colitis. Accumulated CD8$^+$ T cells release proinflammatory cytokines that ultimately disrupt the barrier and increase intestinal permeability by inducing epithelial cell apoptosis.[43,44] This cascade of events perpetuates a vicious cycle of further immune cell recruitment and activation, ultimately leading to sustained tissue injury. Moreover, CXCL9, also known as monokine induced by gamma interferon (MIG), is not only produced in response to IFN. Extracellular ATP can induce elevated CXCL9 expression by activating caspase-1.[45] Perforin- and GzmB-mediated cytotoxicity enhances intestinal mucosal inflammation by inducing the release of ATP from damaged epithelial cells.[46] The mechanisms by which perforin and GzmB upregulate CXCL9 expression in the colon are under further investigation.

Recent studies have identified that anti-depressant therapy may be beneficial in patients with IBD, improving prognosis through both anti-inflammatory effects and somatic symptom control.[47,48] We contemplated the therapeutic potential of anti-CXCL9 antibodies, which could neutralize CXCL9, protect hippocampal neurons, and alleviate IBD-induced depression. Although our data indicated that CXCL9 antibodies primarily improved depression induced by IBD without affecting the severity of IBD, we believed that addressing the psychological state of patients with IBD might also positively impact their overall disease symptoms. Given that IBD is a relapsing condition, bolstering mental health could alleviate the burden of both psychological and gastrointestinal symptoms on patients' lives. Our findings also revealed that CD8$^+$ T-cell–derived perforin and GzmB were key in IBD-associated depression, suggesting potential therapeutic interventions. We proposed the use of inhibitors to target the production of these proteins or antisense oligonucleotides to suppress their expression, thereby mitigating IBD-induced depressive symptoms. Building on this foundation, novel potential applications for these inhibitors and neutralizing antibodies could simultaneously target both IBD and depression induced by intestinal inflammation.

There are limitations in the current study. First, all animal experiments were conducted using male mice exclusively and the potential impact of sex differences remains to be explored. Second, clinical manifestations of anxiety have also been observed in patients with IBD, thus, further investigation into the immunological mechanisms of other emotional disorders related to IBD, such as anxiety, is crucial for advancing our understanding of these conditions.

In conclusion, this study elucidates a potential mechanism by which intestinal inflammation can exacerbate depression. Our findings highlight the novel role of perforin produced by colonic CD8$^+$ T cells in gut-brain communication. This study also identifies perforin and CXCL9 as potential therapeutic targets for psychiatric comorbidities in patients with IBD.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.20110.1053/j.gastro.2025.02.036.

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CRediT Authorship Contributions
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Conflicts of interest
The authors disclose no conflicts.

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Data Availability
The sequencing data from this study can be found at the China National Center for Bioinformation (CNCB) under accession PRJCA026385 and PRJCA026644. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dong Zhang (zhangd@ccmu.edu.cn).