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## Complement component 3a receptor deficiency attenuates chronic stress-induced monocyte infiltration and depressive-like behavior

Amanda Crider<sup>1</sup>, Tami Feng<sup>1</sup>, Chirayu D. Pandya<sup>1</sup>, Talisha Davis<sup>1</sup>, Ashwati Nair<sup>1,2</sup>, Anthony O Ahmed<sup>3</sup>, Babak Baban<sup>4</sup>, Gustavo Turecki<sup>5</sup>, and Anilkumar Pillai<sup>1</sup>

<sup>1</sup>Department of Psychiatry and Health Behavior, Medical College of Georgia, Augusta University, Augusta, GA 30912

<sup>2</sup>Institute of Pharmacy, Nirma University, Ahmedabad, India

<sup>3</sup>Department of Psychiatry, Weill Cornell Medical College, 1300 York Ave, New York, NY 10065

<sup>4</sup>Department of Oral Biology, Dental College of Georgia, Department of Neurology, Augusta University, Augusta, GA 30912

<sup>5</sup>McGill Group for Suicide Studies, Depressive Disorders Program, Douglas Mental Health University Institute, McGill University, Montreal, Quebec, Canada

### Abstract

Major depressive disorder (MDD) is one of the most common and debilitating neuropsychiatric illnesses. Accumulating evidence suggests a potential role of the immune system in the pathophysiology of MDD. The complement system represents one of the major effector mechanisms of the innate immune system, and plays a critical role in inflammation. However, the role of complement components in MDD is not well understood. Here, we found significant increase in component 3 (C3) expression in the prefrontal cortex (PFC) of depressed suicide subjects. We tested the role of altered C3 expression in mouse model of depression and found that increased C3 expression in PFC as a result of chronic stress causes depressive-like behavior. Conversely, mice lacking C3 were resilient to stress-induced depressive-like behavior. Moreover, selective overexpression of C3 in PFC was sufficient to cause depressive-like behavior in mice. We found that C3a (activated product of C3) receptor, C3aR<sup>+</sup> monocytes were infiltrated into PFC following chronic stress. However, C3aR knockout mice displayed significantly reduced monocyte

**Address for Correspondence:** Anilkumar Pillai, Ph.D., Associate Professor, Department of Psychiatry and Health Behavior, Medical College of Georgia, Augusta University, 1120 15th Street, Augusta, GA 30912, Phone – (706) 446-0325, apillai@augusta.edu.

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### Authors' contributions

A.P contributed to study design, data collection, analysis and writing. A.C., T.F., C.D.P., T.D., and A.N. contributed to data collection and analysis. B.B performed flow cytometry analysis. A.O.A contributed to statistical analysis. G.T provided the human postmortem tissues.

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recruitment into PFC and reduced levels of the proinflammatory cytokine IL-1 $\beta$  in PFC after chronic stress. In addition, C3aR knockout mice did not exhibit chronic stress-induced behavior despair. Similarly, chronic stress-induced increases in C3aR+ monocytes and IL-1 $\beta$  in PFC, and depressive-like behavior were attenuated by myeloid cell depletion. These postmortem and preclinical studies identify C3aR signaling as a key factor in MDD pathophysiology.

## Keywords

complement; C3; depression; animals; behavior

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## 1. Introduction

Recent studies from both clinical as well as preclinical studies suggest that immune system plays an important role in the pathophysiology of Major Depressive Disorder (MDD)<sup>1,2</sup>. The role of inflammation in MDD is further supported by studies showing increased levels of pro-inflammatory cytokines in depressed subjects<sup>3,4</sup>. In particular, increased levels of IL-1 $\beta$  mRNA and protein levels have been found in the prefrontal cortex (PFC) of depressed and suicide subjects<sup>5</sup>. The PFC is most sensitive to the detrimental effects of stress exposure, exhibiting both structural as well as functional changes upon chronic stress, and has been especially implicated in depression<sup>6-10</sup>. Chronic stress has been shown to increase microglia activation in PFC, but not in hippocampus<sup>11</sup>. The association between immune activation and depressive behavior is further strengthened by findings from preclinical studies that show induction of depressive-like behaviors in rodents following cytokine administration, and increased proinflammatory signaling in rodent models of depressive-like behavior<sup>12-15</sup>. However, the endogenous primers for this activation are not well understood.

The innate immune system responds by recognition of conserved motifs in pathogens as well as a number of other indicators of cell stress such as cytokines leading to the activation of adaptive immunity<sup>16</sup>. The complement system is a vital component of innate immunity and is one of its major effector mechanisms<sup>17</sup>. Complement consists of a number of inactive components that are activated in a cascade-like manner by three major pathways: the classical pathway, lectin pathway, and alternate pathway<sup>18,19</sup>. All pathways converge upon generation of C3 convertase, a molecule that cleaves complement component C3 into C3a and C3b. C1q is the recognition molecule of the classical pathway, and its binding to antigens or antibodies can activate the associated serine proteases C1r and C1s, leading to cleavage of C2 and C4, which generates the C3 convertase, C4b2b; C4b2b cleaves C3 and activates downstream cascade components. The lectin pathway is initiated by the binding of mannose binding lectin (MBL) to mannose residues on the cell surface. This activates the MBL-associated proteases mannose binding lectin serine protease 1 (MASP1) and MASP2, which then cleave C4 to generate the C3 convertase, C4b2b. The alternative pathway is continuously activated by a “tickover” mechanism (via spontaneous C3 hydrolysis), and functions primarily as an amplification loop of C3b after initiation by the classical pathway and the lectin pathway. C3b opsonizes pathogens to facilitate phagocytosis. C3a, on the other hand, mediates immunomodulatory signals via its seven-transmembrane domain receptor, C3aR<sup>20</sup>.

In the CNS, complement has additional physiological activity outside of immune function. C3 is expressed in neurons<sup>21</sup>, glia<sup>22</sup> and endothelial cells<sup>23</sup>. C3 signaling through C3aR has been shown to regulate synaptic function and dendritic morphology<sup>22</sup>. Complement components enhance proinflammatory TLR-mediated signaling leading to increased production of IL-1 $\beta$ <sup>24</sup>. Despite the intense interest into inflammation in depression, little is known about the role of complement system in depression. In the present study, we examined the role of C3 in depression using postmortem PFC samples from depressed suicide subjects and rodent models of depression. We demonstrate that selective C3 overexpression in the mPFC is sufficient to produce depressive-like behavior. C3 as well as C3aR KO mice were resilient to chronic stress. We found that C3a (activated product of C3) receptor, C3aR+ monocytes were infiltrated into PFC following chronic stress. However, C3aR knockout mice displayed significantly reduced monocyte recruitment into PFC and reduced levels of the proinflammatory cytokine IL-1 $\beta$  in PFC after chronic stress. Together, we identify a novel role of C3 in depressive behavior.

## 2. Materials and Methods

### 2.1. Animals

Adult (8 week old) male C57BL/6J mice were purchased from The Jackson Laboratory. Two month old C3<sup>-/-</sup> (129S4-C3<sup>tm1Crr/J</sup>), C3aR<sup>-/-</sup> (129S4-C3aR<sup>tm1Cge/J</sup>) and their age-matched wild type (WT) controls were purchased from The Jackson Laboratory and housed and maintained in our animal housing facility at Augusta University. Mice were housed in groups of 4 mice in standard polypropylene cages in 12-h light-dark cycle in compliance with the US National Institute of Health guidelines and approved by Augusta University animal welfare guidelines. Mice were assigned to experimental groups based on their genotype. Selection of animal for lentiviral treatment was performed randomly and in a blinded manner.

### 2.2. Stereotaxic Injection of Lentivirus

C3 Lentiviral (LV) Activation Particles and Control LV Activation Particles were purchased from Santa Cruz Biotech, CA, USA. C3 LV Activation Particles contain the following SAM activation elements: a deactivated Cas9 (dCas9) nuclease (D10A and N863A) fused to the transactivation domain VP64, an MS2-p65-HSF1 fusion protein, and a target-specific 20 nt guide RNA. Lentiviral particles frozen stock contains a concentration of  $1.0 \times 10^6$  infectious units of virus in Dulbecco's Modified Eagle's Medium with 25 mM HEPES pH 7.3. Lentiviral particles were injected stereotaxically into mouse PFC<sup>25,26</sup>. In brief, mice were received Buprenorphine (0.05 mg/kg, s.c.) for 15 min and then anesthetized with 1.5–2.5% isoflurane mixed with oxygen in an induction chamber. Mice were maintained under deep anesthesia and mounted in a stereotaxic apparatus. Around ~1 mm size burr hole were made to perform a very small craniotomy as per the recommended coordinates (x .5 mm (lateral), y 1.0 mm (anteriorposterior A–P), with respect to bregma at 0), z 1.0 mm (dorso–ventral D–V with brain surface at 0). A 30–gauge needle attached to a micro syringe containing lentivirus were inserted and lowered into the prefrontal cortical region of the brain. The microsyringe was left in place 5 min after each injection, and a total volume of 1.0  $\mu$ l ( $1 \times 10^6$  infectious particles per milliliter) of lentivirus was administered at a rate of 0.2  $\mu$ l/min at

each site (Stoelting Co). Mice were allowed to recover from anesthesia, transferred to a clean cage with free access to chow diet and water. After the 2 wk of injection, the injection site was confirmed by imaging analysis for the fluorescence of lentiviral particles.

### 2.3. Chronic unpredictable stress (CUS) procedure

Two weeks prior to the beginning of the CUS paradigm, all animals were individually housed while all other aspects of housing remained as standard housing conditions. Mice underwent CUS for 21 days during the light phase of the 24 h period. CUS experiments were performed according to our previously published procedure<sup>26</sup>. Details on the duration and types of stressors used are provided in Table S1. The controls received the same temporal and spatial sequence of habituation and test days that the stressed animals experienced.

### 2.4. Behavioral Tests

Behavioral testing was conducted 24 h following the final period of CUS exposure during the light phase of the 24 h period. Behavior tests were performed in the following sequential order in every animal: (1) open field test, (2) tail suspension test, (3) forced swim test, and (4) sucrose preference test with a period of rest of at least 24 h between each test. Immediately following the sucrose preference test, mice were sacrificed and brains were rapidly removed and processed for biological measures.

**2.4.1. Sucrose preference test**—Mice were habituated to 1% sucrose solution for 3 days at the start of the experiment, in which two bottles of 1% sucrose solution were placed in each cage. After adaptation, mice were deprived of fluid for 16 h. Mice were then presented with two identical bottles of 1% sucrose and water for 24 h. The position of the bottles containing water or sucrose solution was switched halfway through the test. At the end of the 24-h test, sucrose and water consumption (ml) were measured, and the preference for sucrose was calculated as a percentage of consumed sucrose solution of the total amount of liquid drunk.

**2.4.2. Forced swim test**—Mice were subjected to one 5-min session of swimming in water. On the test day, mice were placed in a clear cylinder with water ( $24\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ , 45-cm depth and 20 cm diameter). The sessions were recorded from the side, and the immobility time was scored by a blind observer. Immobility was calculated as the least amount of movement of experimental mice to stay afloat.

**2.4.3. Tail Suspension test**—Each mouse was suspended with a tape 0.75 cm away from their tails and elevated 70 cm above the floor. Behavior was recorded for 6 min. Observer blinded to the subject's treatment group scored video recordings for time spent struggling or immobile.

**2.4.4. Open field test**—Mouse locomotor activity in an open field was measured over a 10-min period. Time spent (sec.) in margin and center, total distance (cm) and mean speed (cm/sec.) were measured during the session. Ethovision XT 10 (Noldus Information Technologies Inc, USA) software and open field chamber ( $40 \times 40 \times 40$  cm), and a video

camera was fixed over the chamber by an adjacent rod, an activity monitor, a programmer and a printer were used for analysis. Sessions total for all parameters were taken. Observations were recorded for 10 min.

## 2.5. Monocyte/macrophage depletion

A Clodrosome Macrophage Depletion Kit, containing control liposomes (Encapsome) and clodronate liposomes (Clodrosome) (Encapsula NanoSciences, Brentwood, TN), was used to deplete endogenous monocytes/macrophages. Intraperitoneal administration of 200  $\mu$ l of Encapsome or clodronate liposomes (5 mg/ml) was performed three times at 7 days apart during CUS paradigm for 21 days. Following CUS, myeloid cell (CD11b<sup>+</sup> F4/80<sup>+</sup>) depletion was confirmed by flow cytometry.

## 2.6. Western blotting

PFC samples collected immediately following decapitation under anesthesia were lysed in ice-cold lysis buffer containing protease inhibitor cocktail (1860932, ThermoFisher scientific, Waltham, MA) and protein concentration was determined by bicinchoninic acid (BCA) assay (MilliporeSigma St. Louis, MO). Protein was electrophoretically separated on a SDS PAGE gel and transferred to a nitrocellulose membrane. Blots were incubated in the appropriate primary antibody specific for C3 (sc-28294, Santa Cruz Biotech, Dallas, Texas), C3aR (sc-133172; Santa Cruz Biotech), VCAM-1 (114441-1-AP; Proteintech, Rosemont, IL), ICAM-1 (10831-1-AP; Proteintech),  $\beta$ -tubulin (2146, Cell Signaling, Danvers, MA) or  $\beta$ -actin (4967, Cell Signaling); and developed with the ECL Plus Western Blotting Detection System (GE Healthcare). Optical densities of the bands were analyzed using ImageJ software (NIH). For analysis, protein levels were normalized to  $\beta$ -tubulin or  $\beta$ -actin levels then expressed as a fold change of that in control animals.

## 2.7. Flow cytometry

Mice were anesthetized with isoflurane and perfused with ice-cold PBS. The brain was removed and the PFC separated and homogenized in cold PBS. Freshly harvested PFC tissue was sieved through a 100- $\mu$ m cell strainer (BD Biosciences, San Diego, CA), followed by centrifugation (1000 rpm, 10 min) to prepare single-cell suspensions. Cells were incubated with Abs against cell surface markers, CD11b, F4/80, CD45 (all from eBioscience, San Diego, CA), Iba-1 (019-19741, Wako Chemicals USA, Inc. Richmond, VA) and C3aR (sc-133172; Santa Cruz Biotech). Following a PBS wash, cells were fixed and permeabilized using a Fixation/Permeabilization Concentrate (Affymetrix eBioscience). Cells were analyzed using a four-color flow cytometer (FACSCalibur) and CellQuest software (both from BD Biosciences, San Jose, CA)<sup>27</sup>. Isotype-matched controls were analyzed to set the appropriate gates for each sample. For each marker, samples were analyzed in duplicate. To minimize false-positive events, the number of double-positive events detected with the isotype controls was subtracted from the number of double-positive cells stained with the corresponding Abs (not isotype control). Cells expressing a specific marker were reported as a percentage of the number of gated events. Mean channel fluorescence intensity (MFI) derived from a fluorescence graph was used to study the level of cell surface C3aR expression.

## 2.8. Quantitative reverse transcriptase PCR (qRT-PCR)

For qRT-PCR analyses, the mouse PFC tissues were collected immediately following decapitation under anesthesia. RNA from mouse as well as human PFC samples were purified using a commercially available kit (SV RNA Isolation, Promega, Madison, WI, USA), qRT-PCR was performed on a MasterCycler (Eppendorf) using a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA). Gene-specific primers were synthesized by Integrated DNA Technologies. Ct values of genes of interest were normalized to that of housekeeping genes ( $\beta$ -actin, ribosomal protein S3 (RPS3) or 18S). Primers used were mC3-FP, 5'-AGCAGGTCATCAAGTCAGGC-3'; mC3-RP, 5'-GATGTAGCTGGTGTGGGCT-3'; mIL1 $\beta$ -FP, 5'-CCAAGCAACGACAAAATACC-3', mIL1 $\beta$ -RP, 5'-GTTGAAGACAAACCGTTTTCC-3' m $\beta$ -actin-FP, 5'-GTTGTGCGACGACGAGCG-3'; m $\beta$ -actin-RP, 5'-AATGAACCGAAGCACACCATA-3'; mRPS3-FP, 5'-GCACAGAGCCTCGCCTT-3'; mRPS3-RP, 5'-ATCAGAGAGTTGACCGCAGTT-3'; hC3-FP, 5'-GCTGCTCCTGCTACTAACCCA-3'; hC3-RP, 5'-AAAGGCAGTCCCTCCACTT-3'; h18S-FP, 5'-GGCCCTGTAATTGGAATGAGTC-3'; h18S-RP, 5'-CCAAGATCCAACACTACGAGCTT-3';

## 2.9. Immunofluorescence staining

Mice were anesthetized with isoflurane and perfused transcardially with PBS followed by 4% paraformaldehyde (PFA). Brains were removed and postfixed in PFA solution overnight and stored in 30% sucrose in PBS until the tissues sank. These were embedded in optimal cutting temperature (OCT) medium, frozen in isopentane ( $-55^{\circ}\text{C}$ ), and stored at  $-80^{\circ}\text{C}$  until use. Serial sections (10  $\mu\text{m}$ ) were cut using a cryostat microtome (Leica CM 3050 S; Leica Microsystems, Chantilly, Virginia) and stored at  $-20^{\circ}\text{C}$ . Sections were thawed, washed twice in PBS for 5 min and were incubated with blocking solution (10% normal donkey serum in PBS) with constant shaking for 30 min followed by overnight incubation with a cocktail of primary antibodies in 5% normal donkey serum in PBS containing 0.3% triton X-100. We used an antibody directed against the ionized calcium-binding adapter molecule-1 (Iba-1; 1:100; 019-19741; Wako) which recognizes both microglia and macrophages<sup>28</sup>. To stain perivascular macrophages<sup>29</sup>, a myeloid cell type that resides in the perivascular space, we used an antibody to mannose receptor CD206 (1:100; ab64693; abcam). The above antibodies were used in combination with antibody to C3 (1:100; sc-28294, Santa Cruz Biotech, or 2934R; Bioss antibodies; Woburn, Massachusetts). After rinsing, sections were incubated for 2 h with Cy3 or Cy2-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, PA) in 5% normal donkey serum in PBS containing 0.3% triton X-100. Sections were thoroughly rinsed, mounted, and cover-slipped in anti-fading aqueous medium. Confocal images were acquired using a Zeiss 510 LSM microscope.

## 2.10. Il-1 $\beta$ protein levels by enzyme linked immunosorbent assay (ELISA)

Mouse PFC samples collected immediately following decapitation under anesthesia were lysed in ice-cold lysis buffer containing protease inhibitor cocktail and protein concentration was determined by BCA assay. Il-1 $\beta$  protein levels in mouse PFC samples were measured



by ELISA according to the manufacturer's instructions (#MLB00C; R&D Systems). The values are expressed as pg/mg protein.

### 2.11. Postmortem samples

Human postmortem PFC tissue samples (Brodmann's area 10) from suicide completers (N=15) and psychiatrically normal controls (N=15) were used in the current study. The samples were obtained from the Quebec Suicide Brain Bank (QSBB; Douglas Institute; [www.douglas.qc.ca/suicide](http://www.douglas.qc.ca/suicide)). The study was granted by the Douglas Hospital Institutional Review Board and, written informed consent was obtained from each participating family (Declaration of Helsinki, 1964).

### 2.10. Statistical Analysis

For mouse studies, data were analyzed using two-tailed Student's t-tests (for two-group comparisons) or Analysis of Variance (ANOVA; for multiple-group comparisons).  $p < 0.05$  was considered significant. Post hoc analyses were carried out using Bonferroni's test. For the postmortem analysis, Analysis of Covariance (ANCOVA) was used to examine differences between depressed-suicide subjects and the controls on C3 expression. Group differences in C3 expression were examined while age, post mortem interval (PMI), pH, RNA integrity, lifetime substance use, substance use at death, and psychiatric medication use were evaluated as possible covariates for inclusion in the model. It was decided *a priori* that candidate covariates with at least small correlations with C3 expression would be included as a covariate in the ANCOVA. The effect size difference between depressed-suicide and control subjects was estimated as partial eta-square ( $\eta^2_p$ ). All analyses were completed using SPSS Statistics 20 software (IBM).

## 3. Results

### 3.1. C3 expression is increased in the prefrontal cortex of depressed subjects

There were no significant differences between postmortem samples obtained from the depressed-suicide sample and controls in any of the evaluated covariates (Table S2 and Table S3). Only lifetime substance use ( $\phi=0.333$ ), substance use at death ( $\phi=0.208$ ), and psychiatric medication use ( $\phi=0.283$ ) were retained as covariates following the initial evaluation. To determine whether C3 expression is altered in depressed subjects, we conducted quantitative PCR analysis of postmortem PFC samples collected from a cohort of suicide victims diagnosed with depression and age-matched individuals without psychiatric diagnoses<sup>26</sup>. We found a significant increase in C3 mRNA levels in postmortem PFC samples obtained from depressed suicide subjects as compared to control subjects (Fig 1;  $P < 0.001$ ). A key limitation in the studies using postmortem samples is the presence and history of substance use and/or medications at the time of death. Out of 15 depressed suicide subjects, 11 had the presence of substance use and 7 had medication at the time of death. To determine if the observed increase in C3 mRNA levels in the PFC of depressed suicide subjects are related to these potential covariates, we performed ANCOVA. None of the three covariates—substance use [ $F(1,25) = 1.709$ ,  $p = 0.203$ ,  $\eta^2_p = 0.064$ ], substance use at death [ $F(1,25) = 0.109$ ,  $p = 0.744$ ,  $\eta^2_p = 0.004$ ], and medication use [ $F(1,25) = 2.126$ ,  $p = 0.157$ ,  $\eta^2_p = 0.078$ ] were significantly associated with C3 mRNA in the ANCOVA. Depressed-

suicide status was a significant predictor of C3 mRNA [ $F(1, 25) = 40.27, p < 0.001, \eta^2_p = 0.617$ , Observed Power = 1.00]. Additional analysis compared the effect of antidepressant treatment on C3 mRNA levels, but no significant effect was observed.

### 3.2. C3 knockout mice are resilient to CUS-induced depressive-like behavior

Because C3 is a converging point of all three complement activation pathways and is highly expressed in the PFC of depressed suicide subjects, we further examined the function of C3 in depressive-like behavior using rodent model of depression. We used a mouse chronic unpredictable stress (CUS) paradigm that is a well validated rodent model of depressive-like behavior<sup>26</sup>. In this paradigm, mice were exposed to a series of mild unpredictable stressors for 21 days. At the end of the stress paradigm, the animals were tested for tail suspension test (TST), forced swim test (FST), open field activity (OF) and sucrose preference test (SPT). The CUS-exposed mice gained significantly less weight over 21 days (Fig 2A;  $p < 0.05$ ). There was a significant increase in C3 mRNA (Fig 2B;  $p < 0.05$ ) and protein (Fig 2C;  $p < 0.05$ ) levels in PFC of CUS-exposed mice, compared to non-stressed (NS) mice. Immunofluorescence analysis of PFC sections from CUS-exposed mice showed that C3 is colocalized with Iba-1, a marker for myeloid cells (monocyte/macrophage lineage) and CD206, a marker for perivascular macrophages, a myeloid cell type that resides in the perivascular space (Fig 2D).

To further examine the role of C3 in depressive-like behavior, we used C3 KO mice. Our baseline behavioral analysis (before exposure to CUS) showed no significant difference between C3 KO and WT mice in TST, FST, OF and SPT (data not shown). Next, we examined whether deletion of C3 influences the response to CUS. As expected CUS exposed WT animals showed significant increases in immobility time in TST (Fig 2E) and FST (Fig 2F). However, the immobility time in C3 KO mice exposed to CUS were similar to WT mice in both TST (Fig 2E; two-way ANOVA, stress,  $F_{(1,24)} = 15.06, p < 0.001$ ; genotype,  $F_{(1,24)} = 7.84, p < 0.01$ ; stress  $\times$  genotype,  $F_{(1,24)} = 5.12, p < 0.05$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.001$ , CUS-WT vs CUS-C3 KO,  $p < 0.001$ ) and FST (Fig 2F; two-way ANOVA, stress  $\times$  genotype,  $F_{(1,24)} = 6.50, p < 0.05$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.05$ , CUS-WT vs CUS-C3 KO,  $p < 0.05$ ). In sucrose preference test, CUS resulted in a significant reduction in sucrose consumption in WT mice, indicative of stress-induced anhedonia (Fig 2G). However, C3 KO mice exposed to CUS consumed sucrose volumes that were similar to the levels in WT group (Fig 2G; two-way ANOVA, stress,  $F_{(1,24)} = 5.61, p < 0.05$ ; genotype,  $F_{(1,24)} = 7.28, p < 0.05$ ; stress  $\times$  genotype,  $F_{(1,24)} = 4.50, p < 0.05$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.01$ , CUS-WT vs CUS-C3 KO,  $p < 0.01$ ). We did not find any significant effect on locomotor activity in the open field test (Fig 2H). These results demonstrate that C3 KO mice show normal behavior in the absence of stress, but are resistant to CUS-induced depressive-like behavior.

### 3.3. Overexpression of C3 in PFC causes depressive-like behavior in mice

To determine whether restricting C3 overexpression to a pathologically relevant brain region would suffice to create the behavioral deficits, we infused mice with control or C3 lentiviral activation particles in the medial PFC (mPFC) by a lentiviral approach (Fig. 3A). Following two weeks to allow for expression of viral constructs, mice were tested in measures of



depressive-like behavior. We found significant increase in C3 mRNA expression in PFC following C3 administration (Fig 3B;  $p < 0.05$ ). In behavior tests, mice infused with C3 demonstrated significant increases in immobility time in TST (Fig 3C;  $p < 0.05$ ) and FST (Fig 3D;  $p < 0.05$ ). C3 overexpression resulted in a significant reduction in sucrose consumption compared with control mice (Fig 3E;  $p < 0.05$ ). No significant difference in locomotor activity was observed between the two groups (Fig 3F;  $p < 0.05$ ) suggesting that change in immobility is not due to a generalized decrease in ambulation.

#### 3.4. C3aR knockout mice are resilient to CUS-induced despair behavior

It is known that C3a (activated product of C3) induces immunomodulatory signals via its receptor, C3aR<sup>20</sup>. We found a significant increase in C3aR protein levels in PFC of CUS-exposed mice, compared to non-stressed mice (Fig 4A;  $p < 0.05$ ). To further determine the role of C3aR in depressive-like behavior, we used C3aR KO mice. We found significant increases in immobility time in tail suspension and forced swim tests in WT mice following CUS as compared to non-stressed (NS) mice (Fig 4B–C). However, the immobility time in C3aR KO mice exposed to CUS were similar to WT mice in both TST (Fig 4B; two-way ANOVA, stress  $\times$  genotype,  $F_{(1,20)} = 6.18$ ,  $p < 0.05$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.05$ , NS-WT vs CUS-C3aR KO,  $p > 0.05$ ) and FST (Fig 4C; two-way ANOVA, stress,  $F_{(1,21)} = 5.49$ ,  $p < 0.05$ ; genotype,  $F_{(1,21)} = 4.55$ ,  $p < 0.05$ ; stress  $\times$  genotype,  $F_{(1,21)} = 10.03$ ,  $p < 0.01$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.05$ , NS-WT vs CUS-C3aR KO,  $p > 0.05$ ). In sucrose preference test, CUS failed to induce anhedonia-like behavior in both WT and C3aR KO mice (Fig 4D). In the open field test, CUS exposed WT as well as C3aR KO mice showed hyperactivity as determined by the distance travelled in the chamber (Fig 4E; two-way ANOVA, stress,  $F_{(1,25)} = 23.12$ ,  $p < 0.001$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.001$ , NS-C3aR KO vs CUS-C3aR KO,  $p < 0.05$ ). These results demonstrate that C3aR KO mice are resistant to CUS-induced despair behavior.

#### 3.5. C3aR deficiency reduces the number of chronic stress-induced infiltrating monocytes into PFC

We used flow cytometry analysis to identify the cell types expressing C3aR in PFC following CUS. Differences in the levels of CD45 expression was used to determine if ionized calcium binding adapter molecule 1 (Iba1)-positive cells are infiltrating monocytes from the peripheral circulation, which show high levels of CD45, or part of a resident microglial population that express CD45 at low levels. We found that CUS increased the proportion of Iba1<sup>+</sup>C3aR<sup>+</sup> cells expressing CD45<sup>hi</sup> in PFC (Fig 4F,G).

If C3aR mediates the recruitment of monocytes into PFC of CUS-exposed mice, then in the absence of C3aR we would expect monocyte infiltration to be significantly reduced. Therefore, we examined monocyte migration into PFC of C3aR KO mice following CUS. We found that in CUS exposed C3aR KO mice, the number of Iba1<sup>+</sup>CD45<sup>hi</sup> cells (i.e., infiltrating monocytes) in PFC was significantly reduced compared with CUS exposed WT mice (Fig 4H; two-way ANOVA, stress,  $F_{(1,12)} = 63.74$ ,  $p < 0.001$ ; genotype,  $F_{(1,12)} = 40.38$ ,  $p < 0.001$ ; stress  $\times$  genotype,  $F_{(1,12)} = 22.33$ ,  $p < 0.001$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.001$ , NS-C3aR KO vs CUS-C3aR KO,  $p > 0.05$ ). Also, we found a significant increase in the number of resident microglial cells (Iba1<sup>+</sup>CD45<sup>lo</sup>) in PFC following CUS (Fig 4I; two-

way ANOVA, stress,  $F_{(1,12)} = 33.32$ ,  $p < 0.001$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.001$ , NS-C3aR KO vs CUS-C3aR KO,  $p > 0.05$ ). However, C3aR deficiency did not result in any significant change in the number of Iba1<sup>+</sup>CD45<sup>lo</sup> cells in PFC following CUS. These results indicate that C3aR<sup>+</sup> monocytes infiltrate the PFC following CUS and demonstrate that CUS-induced infiltration of monocytes is C3aR-dependent.

### 3.6. C3aR deficiency reduces CUS-induced increase in VCAM-1, ICAM-1 and IL-1 $\beta$ levels

We examined the protein levels of two key monocyte cell adhesion molecules, VCAM-1 and ICAM-1 which have been well implicated in monocyte infiltration into brain following stress<sup>30</sup>. We found a significant increase in the short isoform of VCAM-1 (~50 KDa) in the PFC of WT mice following CUS [Fig 5A,B; two-way ANOVA, stress,  $F_{(1,10)} = 45.07$ ,  $p < 0.001$ ; genotype,  $F_{(1,10)} = 46.56$ ,  $p < 0.001$ ; stress  $\times$  genotype,  $F_{(1,10)} = 70.12$ ,  $p < 0.001$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.001$ , NS-C3aR KO vs CUS-C3aR KO,  $p > 0.05$ ]. However, the increase of VCAM-1 was attenuated in the C3aR KO mice exposed to CUS compared with WT mice subjected to CUS. Similarly, C3aR deficiency significantly attenuated CUS-induced increase in ICAM-1 protein levels in mouse PFC [Fig 5A,C; two-way ANOVA, stress,  $F_{(1,10)} = 8.21$ ,  $p < 0.05$ ; genotype,  $F_{(1,10)} = 27.28$ ,  $p < 0.001$ ; stress  $\times$  genotype,  $F_{(1,10)} = 39.67$ ,  $p < 0.001$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.001$ , NS-C3aR KO vs CUS-C3aR KO,  $p > 0.05$ ]. No change in the levels of long isoform of VCAM-1 (~100 KDa) was found in any of the treatment groups (Fig 5). To further understand the role of C3aR<sup>+</sup> monocytes in CUS-induced inflammation, we investigated the expression of IL-1 $\beta$ , a key inflammatory mediator in chronic stress conditions in WT and C3aR KO mice at 3 weeks following CUS, when C3aR<sup>+</sup> monocytes have infiltrated PFC. Significant increases in IL-1 $\beta$  mRNA [Fig 5D; two-way ANOVA, stress  $\times$  genotype,  $F_{(1,11)} = 12.24$ ,  $p < 0.05$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.05$ , NS-C3aR KO vs CUS-C3aR KO,  $p > 0.05$ ] and protein [Fig 5E; two-way ANOVA, stress,  $F_{(1,8)} = 8.49$ ,  $p < 0.01$ ; genotype,  $F_{(1,8)} = 22.61$ ,  $p < 0.001$ ; *post hoc*, NS-WT vs CUS-WT,  $p < 0.05$ , NS-C3aR KO vs CUS-C3aR KO,  $p > 0.05$ ] levels were found in PFC of CUS-exposed WT mice. However, the induction of IL-1 $\beta$  was attenuated in the C3aR KO mice exposed to CUS compared with WT mice subjected to CUS (Fig. 5D–E).

### 3.7. Monocyte/macrophage cell depletion attenuates CUS-induced depressive-like behavior

Trafficking of monocytes/macrophages into the CNS is a pathological feature of stress-induced behavioral changes in rodent models<sup>31</sup>. To test whether the infiltration of monocyte-macrophages into PFC contributes to the development of CUS-induced depressive-like behavior, we depleted mice of these myeloid cells by three administrations of liposome-encapsulated clodronate (Clodrosome) at 7 days apart during the CUS paradigm. Administration of Clodrosome significantly reduced the number of circulating CD11b<sup>+</sup>F4/80<sup>+</sup> myeloid cells (Fig. 6A) compared with Encapsome-treated (CON) mice. CUS significantly increased the number of Iba1<sup>+</sup>/C3aR<sup>+</sup>/CD45<sup>hi</sup> cells in PFC in Encapsome-treated mice. However, Clodrosome administration completely inhibited the CUS-induced increase in Iba1<sup>+</sup>/C3aR<sup>+</sup>/CD45<sup>hi</sup> cells in PFC (Fig 6B,C; two-way ANOVA, stress,  $F_{(1,13)} = 130.3$ ,  $p < 0.001$ ; treatment,  $F_{(1,13)} = 33.28$ ,  $p < 0.001$ ; stress  $\times$  treatment,  $F_{(1,13)} = 74.89$ ,  $p < 0.001$ ; *post hoc*, NS-CON vs CUS-CON,  $p < 0.001$ , NS-CON vs CUS-CL,  $p > 0.05$ ).

Clodrosome administration significantly attenuated CUS-induced despair behavior in TST (Fig 6D; two-way ANOVA, stress,  $F_{(1,20)} = 6.46$ ,  $p < 0.05$ ; *post hoc*, NS-CON vs CUS-CON,  $p < 0.01$ , NS-CON vs CUS-CL,  $p > 0.05$ ) and FST (Fig 6E; two-way ANOVA, stress,  $F_{(1,20)} = 11.38$ ,  $p < 0.01$ ; treatment,  $F_{(1,20)} = 31.09$ ,  $p < 0.001$ ; stress  $\times$  treatment,  $F_{(1,20)} = 25.69$ ,  $p < 0.001$ ; *post hoc*, NS-CON vs CUS-CON,  $p < 0.001$ , NS-CON vs CUS-CL,  $p > 0.05$ ), and anhedonia-like behavior in SPT (Fig 6F; two-way ANOVA, stress,  $F_{(1,18)} = 8.67$ ,  $p < 0.01$ ; treatment,  $F_{(1,18)} = 4.82$ ,  $p < 0.05$ ; *post hoc*, NS-CON vs CUS-CON,  $p < 0.01$ , NS-CON vs CUS-CL,  $p > 0.05$ ) as compared to CUS-exposed Encapsome-treated mice. No change in the locomotor activity was observed in the open field test between groups (Fig 6G). These results suggest that infiltrating monocyte-macrophages play a critical role in CUS-induced depressive-like behavior.

#### 4. Discussion

Our results demonstrate that increased C3 signaling induces depressive-like behavior in the TST and FST, and is sufficient to cause anhedonic behavior in the absence of chronic stress. Conversely, inhibition of C3 signaling using C3 or C3aR KO mice blocks the depressive-like behavior caused by CUS exposure, suggesting resilience to chronic stress. Moreover, C3aR deficiency was sufficient to attenuate stress-induced infiltration of monocytes and IL-1 $\beta$  mRNA levels in PFC. Together, these findings suggest that altered C3 signaling is a potential molecular mechanism underlying the pathology of MDD.

In the brain, C3 may have a role in non-immune neuronal functions<sup>23,32,33,34</sup>. More specifically, studies in C3 knockout mice suggest C3's involvement in the induction of behavioral deficits and regulation of synapses. C3 KO mice demonstrate resiliency towards anxiety and protection against age-related cognitive declines in learning and spatial memory<sup>35,36</sup>, as well as an excessive number of synapses<sup>37,38</sup> and increased synaptic efficacy in response to a high-frequency burst<sup>46</sup>. C3 signaling through C3aR has been shown to regulate synaptic function and dendritic morphology<sup>22</sup>. C3a, the cleaved form of C3, has been implicated in recruiting microglia to synaptically enriched regions<sup>39</sup>. Thus, increased function of the C3 signaling pathway could contribute to chronic stress-induced loss of synapses and impairments in synaptic connectivity in PFC<sup>40,41,42</sup>. Our data on the increased expression of C3 in postmortem PFC of depressed subjects support this possibility and indicate the possible role of C3 in the loss of synapses seen in depression<sup>43</sup>. The increase in C3 expression following chronic stress conditions and attenuation of stress-induced depressive behavior in C3 KO mice as well as in C3aR KO mice further substantiates the role of C3 signaling in depressive-behavior.

The PFC is a highly vulnerable region in response to stress, undergoing structural, neurochemical and functional alterations that determine the deficits in PFC-mediated behaviors<sup>44</sup>. The increase in Iba1+/CD45<sup>hi</sup> cells in the PFC after CUS indicates the recruitment of the infiltrating monocytes. Mice lacking C3aR exhibited complete prevention of the recruitment of monocytes into PFC, indicating that the reduced number of infiltrating monocytes is attributed mainly to the absence of C3aR+ monocyte infiltration. These findings suggest that complement component links the central and peripheral immune systems. Indeed, C3a has been shown to enhance recruitment of peripheral immune cells to

the brain leading to increased neuroinflammation<sup>45</sup>. Moreover, C3 KO and C3aR KO mice demonstrated a reduced amount of infiltrating peripheral cells in the brain compared to wild-type mice<sup>46</sup>. The reduction was attributed to decreased brain endothelial expression of vascular adhesion factors such as VCAM-1 and ICAM-1, which have also been shown as necessary for increased monocyte recruitment during social defeat stress<sup>30,46</sup>. Our data on VCAM-1 and ICAM-1 further suggest the roles of these molecules in monocyte recruitment into PFC following chronic stress. It is known that the trafficking and recruitment of monocytes into brain depends on chemokine receptors. In a mouse model of repeated social defeat (RSD) stress, CCR2<sup>KO</sup> and CX<sub>3</sub>CR1<sup>KO</sup> mice did not exhibit increased brain macrophages following RSD<sup>47</sup>. Moreover, RSD increased CCL2 expression in the brain<sup>47</sup>. Also, CCR2 knockout mice showed reduced monocyte recruitment into brain and reduced levels of the proinflammatory cytokine IL-1 $\beta$  in hippocampus after status epilepticus<sup>48</sup>. Although C3aR signaling has been shown to induce IL-1 $\beta$ , the role of chemokines and their receptors in C3aR-mediated monocyte infiltration and depressive-like behavior following chronic stress are not known, which needs further investigation.

Equally as important as the link between complement and monocytes is the link between complement and microglia. Increased activation of microglia has been found in postmortem brain tissues from depressed and suicide subjects<sup>49,50</sup>, and increased microglial activity in PFC was found to be positively correlated with the severity of depression<sup>51</sup>. In rodents, chronic stress has been shown to increase microglial activation in PFC of mice susceptible to anhedonia<sup>52</sup>. Microglia are the only native cell type expressing C3aR in the brain<sup>53</sup>, and are major sources of CNS-derived IL-1 $\beta$ <sup>54</sup>. A number of studies indicate that the activation of innate immune mechanisms, in particular proinflammatory cytokines IL-1 $\beta$ , IL-6 and tumor necrosis factor alpha (TNF- $\alpha$ ) plays an important role in the pathophysiology of depression<sup>55</sup>. A recent study has found significant increases in IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the PFC of adult depressed suicide victims<sup>56</sup>. C3 enhances proinflammatory signaling and increases production of IL-1 $\beta$ <sup>57</sup>. Our findings on increased C3 mRNA in the PFC of depressed suicide subjects further strengthen the role of innate immune system in depression. In addition, IL-1 $\beta$  inhibition by pharmacological or genetic approach has been shown to attenuate chronic stress-induced depressive behavior in rodents<sup>14,58,59</sup>. We identified Iba1+ myeloid cells and CD206+ perivascular macrophages as the major cellular sources of C3 after CUS. We found that C3aR deficiency is associated with a significant attenuation of CUS-induced increase in IL-1 $\beta$  in the PFC. Additionally, NLRP3 inflammasome activation mediates IL-1 $\beta$ -related inflammation in the PFC of depressive rats<sup>59</sup> and is required for complement-induced IL-1 $\beta$  release<sup>57</sup>. It is known that chronic stress causes an increase in hippocampal astrocyte release of extracellular ATP, which can activate the inflammasome via the receptor P2X7R<sup>60</sup>. Notably, the release of ATP connects C3a and the inflammasome; C3a has been found to bind to C3aR on monocytes and induce release of ATP into the extracellular space upon LPS stimulation *in vitro*<sup>57</sup>. It is possible that this paradigm is replicated in the brain; however, the question of source would then come into play, as microglia as well as invading peripheral monocytes express C3aR. Our data showing inhibition in CUS-induced increase in both the number of C3aR+ monocytes in PFC and depressive-like behavior in Clodrosome-treated mice suggest that C3aR present in peripheral monocytes may play a critical role in chronic stress-induced depressive-like

behavior. It is important to note that monocyte secretion of IL-1 $\beta$  and activation of IL-1R1 on the endothelial surface has been shown as necessary for the development of anxiety behaviors<sup>46</sup>. Therefore, the source of ATP, the identity of the cells expressing C3aR, and the identity of the effector cells warrant further studies in the context of chronic stress.

C3 is regulated in an NF $\kappa$ B-dependent manner, as the C3 promoter contains two putative  $\kappa$ B binding sites<sup>22</sup>. Chronic stress has been shown to induce the activation of NF- $\kappa$ B signaling pathway, which subsequently exerts antineurogenic and anhedonic effects<sup>15</sup>. In addition, NF- $\kappa$ B signaling induces expression of the NLRP3 inflammasome<sup>61</sup>. C3 cleavage can either lead to formation of the downstream MAC, or allow C3a to bind to monocytes to cause ATP release, both of which activate the inflammasome<sup>62</sup>.

In conclusion, our data from human postmortem brain samples and animal studies demonstrate an important role for C3 in mediating depressive behaviors. The high number of covariates in a relatively small cohort of human samples is a limitation in our study. Additional studies using more number of subjects with/without antidepressant medication would be of great interest. It is also important to determine whether CUS induces monocyte infiltration to brain regions other than PFC such as hippocampus and amygdala which are also implicated in depressive-like behavior. Moreover, it is likely that C3 as well as C3aR KO mice show alterations in peripheral monocytes that could impact on immune-to-brain communication following chronic stress and warrants further investigation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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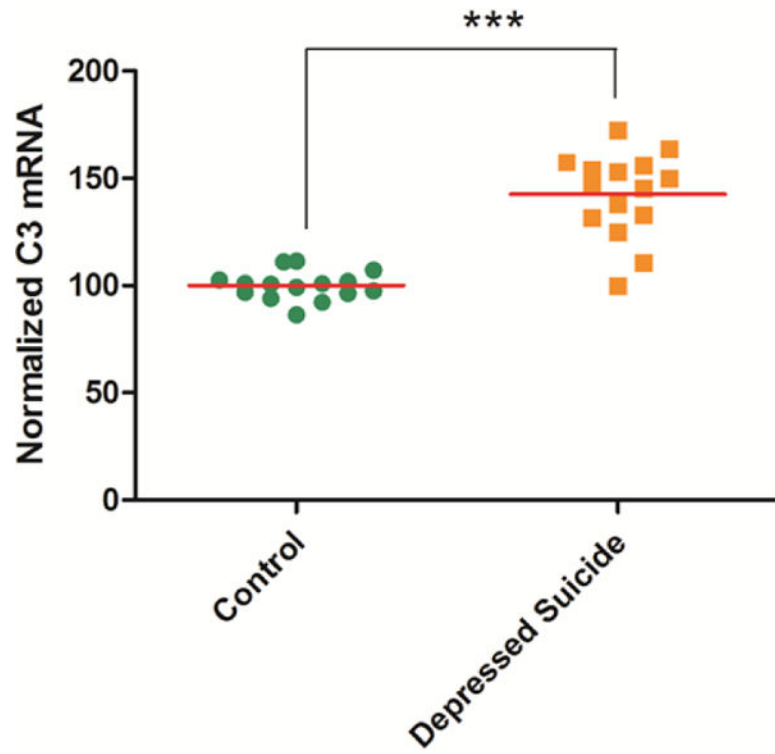


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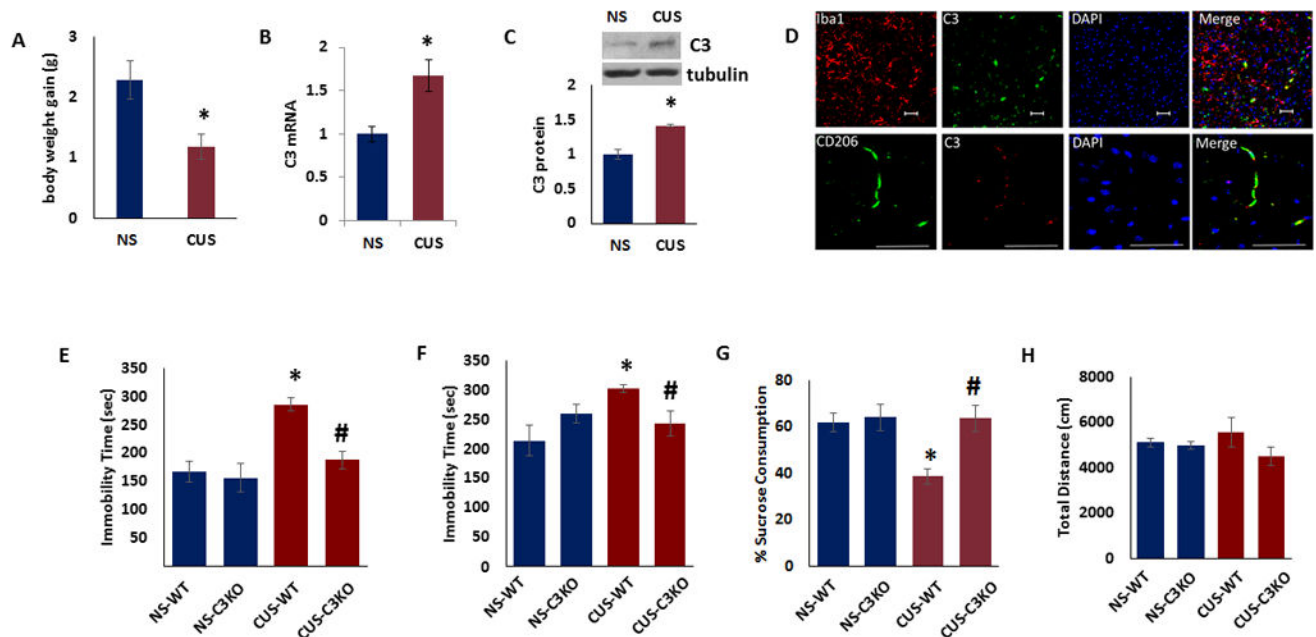
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**Highlights**

- Complement component 3 (C3) mediates chronic stress-induced depressive-like behavior in mice.
- C3a receptor (C3aR) knockout mice were resilient to stress-induced depressive-like behavior.
- C3aR knockout mice displayed significantly reduced monocyte recruitment into PFC after stress.
- Myeloid cell depletion attenuated stress-induced C3aR+ monocyte infiltration into PFC.

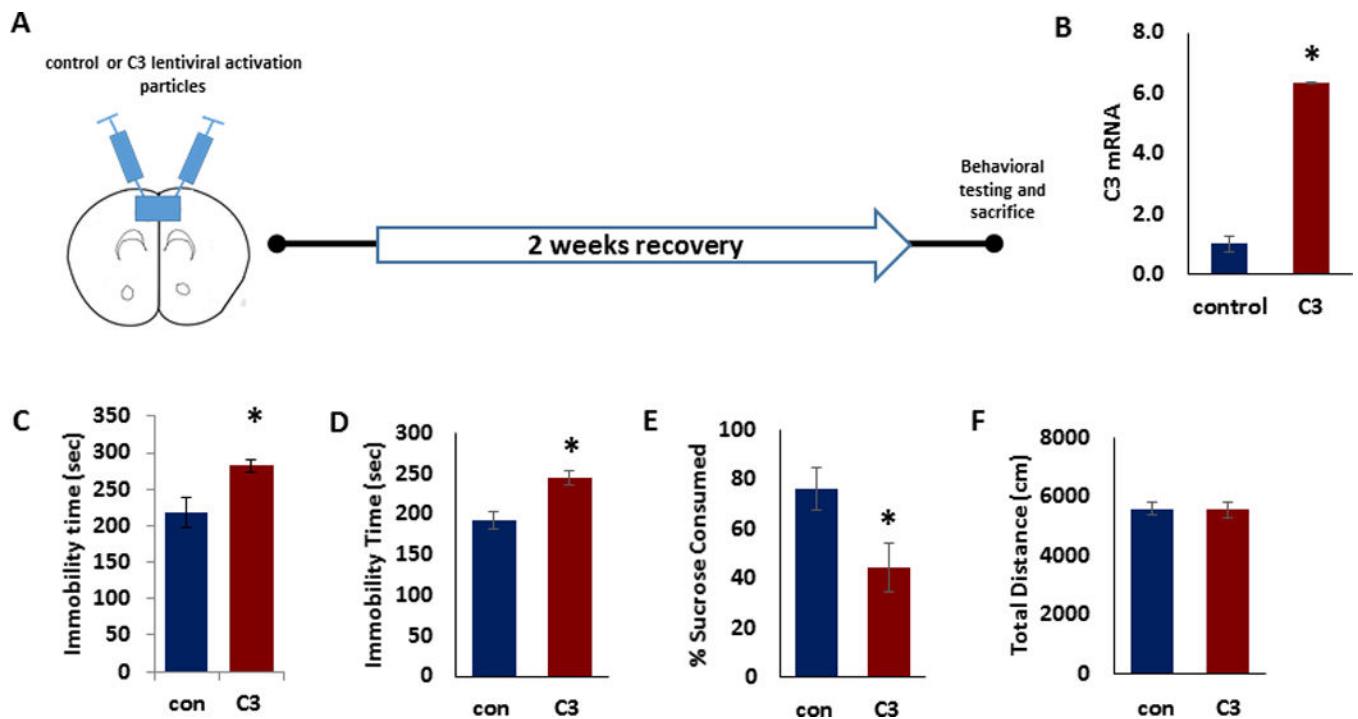


**Figure 1. Increase in C3 mRNA levels in the prefrontal cortex of depressed suicide subjects** C3 mRNA levels in the prefrontal cortex of depressed suicide ( $n=15$ ) and control ( $n=15$ ) subjects were measured by RT-PCR. mRNA levels were normalized to housekeeping gene 18S. \*\*\* $p<0.001$ .



**Figure 2. C3 knockout mice are resilient to CUS-induced depressive-like behavior**

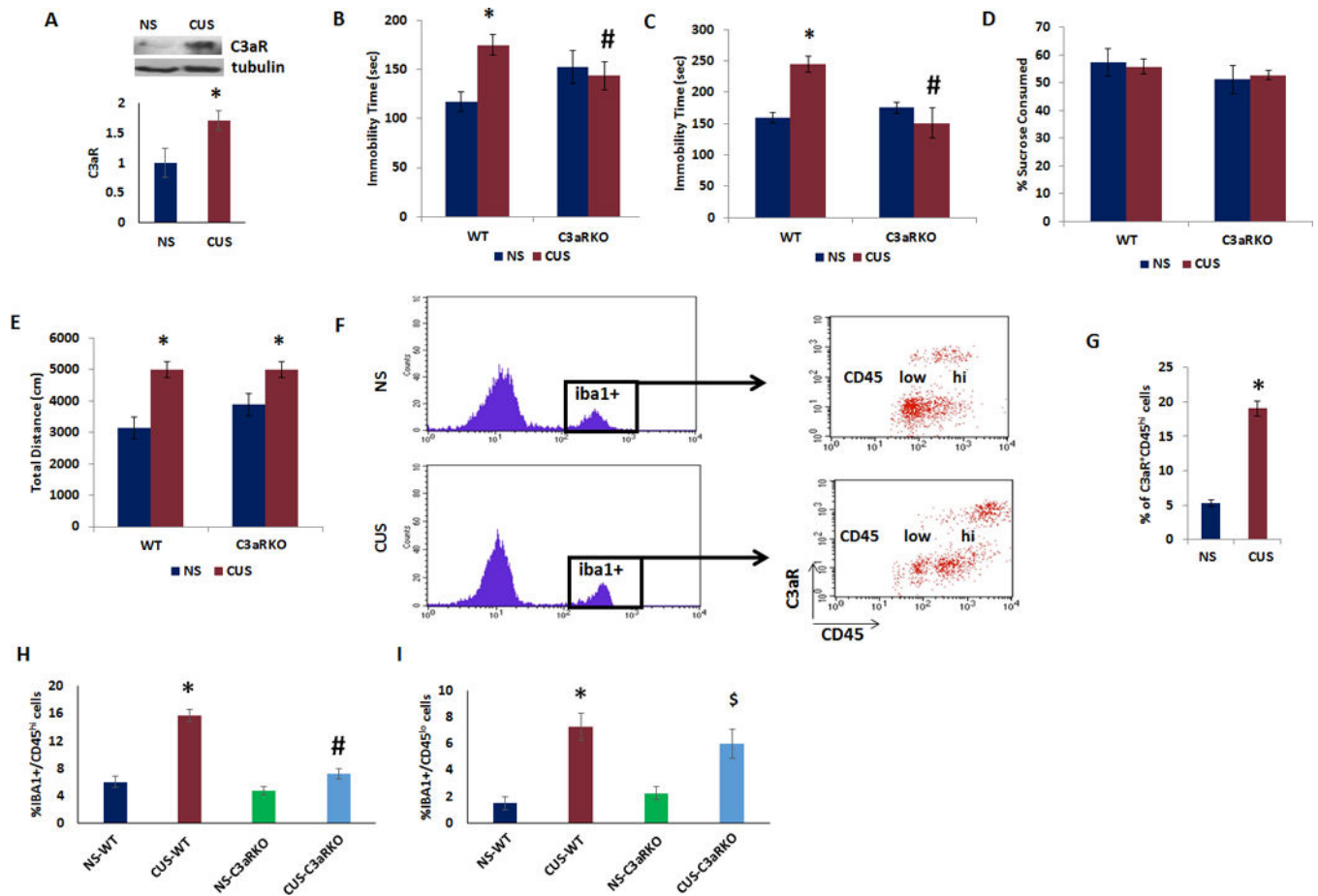
(a) Body weight gain for male mice exposed to CUS ( $n=6$ ) or no stress (NS) ( $n=6$ ) over 21 days. (b) C3 mRNA in the prefrontal cortex of male mice exposed to CUS ( $n=6$ ) or no stress (NS) ( $n=6$ ). The Ct values were normalized to RPS3. (c) *Top*. Representative immunoblot data showing C3 and  $\beta$ -tubulin expression in the PFC of male mice exposed to CUS ( $n=3$ ) or no stress (NS) ( $n=5$ ). *Bottom*. C3 protein levels normalized to  $\beta$ -tubulin. (d) Iba1+ myeloid cells and perivascular macrophages express C3 after CUS. Representative images showing Iba1, and CD206 (perivascular macrophages) staining with C3. (Scale bar: 50  $\mu$ m.) (e–h) Adult male C3 knockout and wildtype (WT) mice were exposed to CUS and depressive-like behavior was determined (N=6–7). (e) Tail suspension test, (f) forced swim test, (g) preference for sucrose in sucrose preference test and (h) distance traveled in the open field test. Data are expressed as mean  $\pm$  s.e.m. \* $P < 0.05$  vs NS (a,b,c) or NS-WT (e–g); # $P < 0.05$  vs CUS-WT (e–g); Student's  $t$  test (a,b,c); two-way ANOVA (e–h).



**Figure 3. Overexpression of C3 in PFC causes depressive-like behavior in mice**

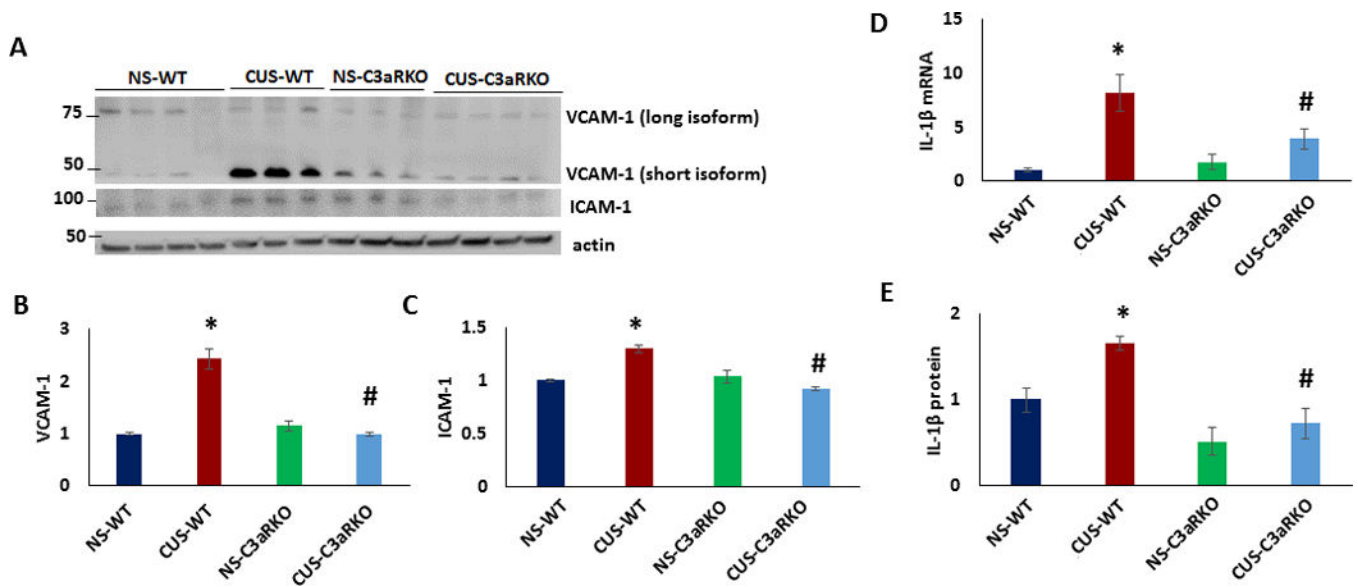
(a) Schematic representation of stereotaxic injection of control (con) or C3 lentiviral activation particles into mouse PFC followed by behavior tests. (b) C3 mRNA in the PFC of mice injected with control or C3 activation particles ( $n=6$ ). The Ct values were normalized to RPS3. (c) Tail suspension test, (d) forced swim test, (e) preference for sucrose in sucrose preference test and (f) distance traveled in the open field test for the mice injected with control or C3 activation particles ( $n=6-8$ ). Data are expressed as mean  $\pm$  s.e.m. \* $P < 0.05$  vs control; Student's  $t$  test.





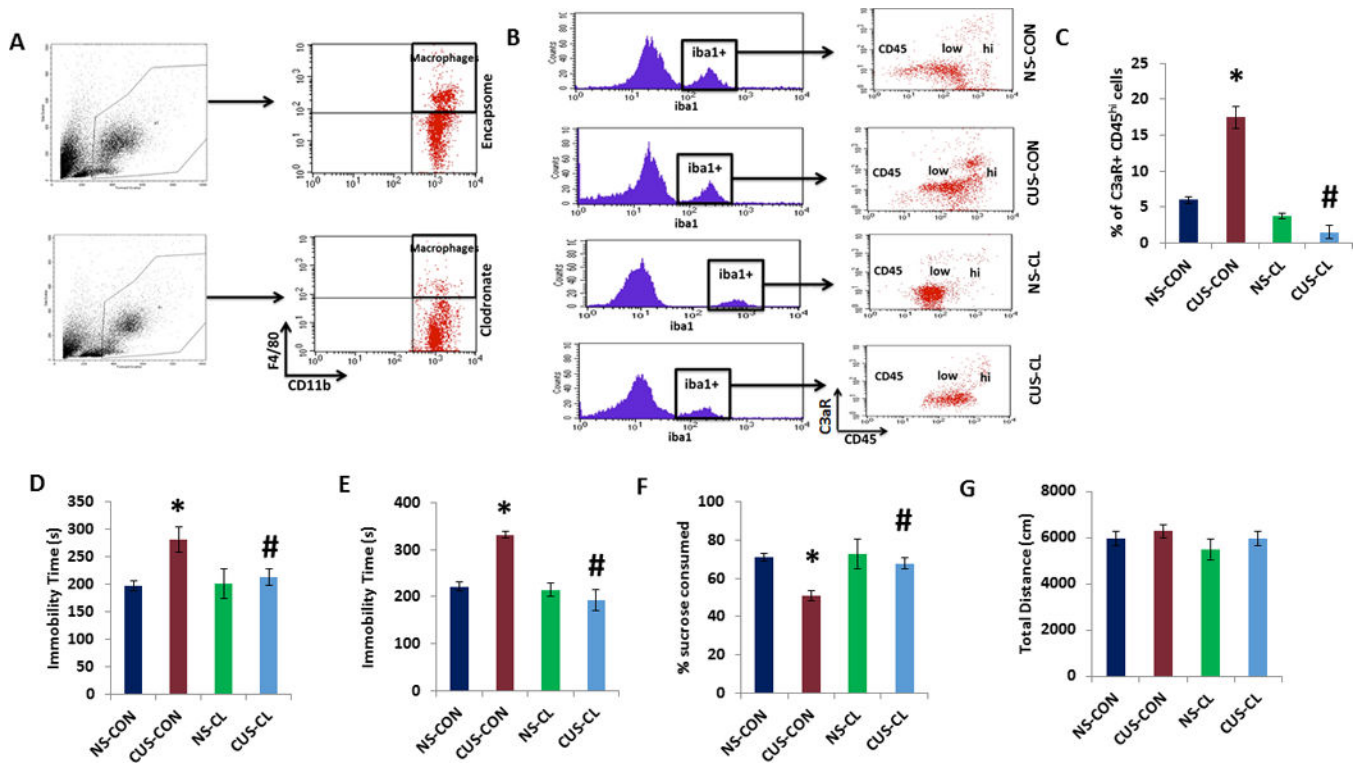
**Figure 4. C3aR deficiency attenuates CUS-induced depressive-like behavior and increase in infiltration of monocytes in PFC**

(a) *Top*. Representative immunoblot data showing C3aR and  $\beta$ -tubulin expression in the PFC of male mice exposed to CUS ( $n=3$ ) or no stress (NS) ( $n=3$ ). *Bottom*. C3aR protein levels normalized to  $\beta$ -tubulin. Adult male C3aR knockout and wildtype (WT) mice were exposed to CUS or no stress (NS) and depressive-like behavior was determined. (b) Tail suspension test, (c) forced swim test, and (d) preference for sucrose in sucrose preference test and (e) open field test. Data are expressed as mean  $\pm$  s.e.m. \* $P < 0.05$  vs NS-WT; # $P < 0.05$  vs CUS-WT; two-way ANOVA.  $N=8$  per group. (f) Adult male mice were exposed to CUS or no stress (NS) and PFC tissues were collected for flow cytometry analysis. Iba1+/C3aR+ cells were analyzed using CD45 to differentiate infiltrating monocytes (CD45<sup>hi</sup>) from resident microglia (CD45<sup>lo</sup>). (g) % of Iba1+ cells expressing C3aR and CD45<sup>hi</sup> in PFC of mice exposed to CUS or no stress (NS). Data are expressed as mean  $\pm$  s.e.m. \* $P < 0.05$  vs NS.  $N=4$  per group. (h-i) Adult male C3aR knockout and wildtype (WT) mice were exposed to CUS or no stress and PFC tissues were collected for flow cytometry analysis. Iba1+ cells were analyzed using CD45 to differentiate (h) infiltrating monocytes (CD45<sup>hi</sup>) from (i) resident microglia (CD45<sup>lo</sup>). \* $P < 0.05$  vs NS-WT; # $P < 0.05$  vs CUS-WT; \$ $P < 0.05$  vs NS-C3aRKO; two-way ANOVA.  $N=4$  per group.



**Figure 5. C3aR deficiency attenuates CUS-induced increase in VCAM-1, ICAM-1 and IL-1 $\beta$  levels in PFC**

PFC tissues from C3aR knockout and WT mice exposed to CUS or no stress (NS) were analyzed for VCAM-1, ICAM-1 and IL-1 $\beta$  levels. (a) Representative immunoblot data showing VCAM-1, ICAM-1 and  $\beta$ -actin expression in the PFC. (b) VCAM-1 (short isoform) and (c) ICAM-1 protein levels normalized to  $\beta$ -actin. (d) IL-1 $\beta$  mRNA in the PFC. The Ct values were normalized to RPS3. (e) IL-1 $\beta$  protein levels were determined by ELISA. Data are expressed as mean  $\pm$  s.e.m. \* $P$  < 0.05 vs NS-WT; # $P$  < 0.05 CUS-WT; Two-way ANOVA. N=3–5 per group.



**Figure 6. Monocyte/macrophage cell depletion attenuates CUS-induced depressive-like behavior** (a) Adult male mice were treated with three administrations of liposome-encapsulated clodronate (Clodrosome; CL) or Encapsome (control; CON) at 7 days apart during the CUS paradigm of 21 days. At the end of the CUS procedure, mice were tested for depressive-like behavior, and PFC samples were collected for flow cytometry analysis. (a) Myeloid cells (CD11b+F4/80+) cells quantified by flow cytometry. (b) Iba1+/C3aR+ cells were analyzed using CD45 to differentiate infiltrating monocytes (CD45<sup>hi</sup>) from resident microglia (CD45<sup>lo</sup>). (c) % of Iba1+ cells expressing C3aR and CD45<sup>hi</sup> in PFC. Data are expressed as mean  $\pm$  s.e.m. \* $P$  < 0.05 vs CON; # $P$  < 0.05 vs CON+CUS; two-way ANOVA. N=4 per group. (d) Tail suspension test, (e) forced swim test, (f) preference for sucrose measured in sucrose preference test, and (g) open field test. Data are expressed as mean  $\pm$  s.e.m. \* $P$  < 0.05 vs NS-CON; # $P$  < 0.05 vs CUS-CON; two-way ANOVA. N=8 per group.