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Identification of a signalling mechanism by which the microbiome regulates Th17 cell-mediated depressive-like behaviors in mice

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Author contributions

EB and SD designed the study. EMMR, DM, GO, DH, MO and EB performed the experiments and analyzed the data. EB, EMMR, SD and SD wrote the manuscript. BLM, MHT provided human samples and CBN contributed to the interpretation of the human data.

Declaration of Interests

The authors declare no conflicts of interest.

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Abstract

Microbiota dysbiosis has been linked to major depressive disorder, but the mechanisms whereby the microbiota modulates mood remain poorly understood. We show that Segmented filamentous bacteria (SFB)-deficient mice are resilient to the induction of depressive-like behavior, and are re-sensitized when SFB is reintroduced in the gut. SFB produces the quorum sensing molecule autoinducer-2 (AI-2) and promotes the production of serum amyloid protein-1 (SAA1) and SAA2 by the host, which increased Th17 cell production. Th17 cells were required to promote depressive-like behaviors by AI-2, as AI-2 administration did not promote susceptibility to depressive-like behaviors or SAA1 and SAA2 production in Th17-deficient mice after stress. Oleic acid, an AI-2 inhibitor, exhibited antidepressant properties, reducing depressive-like behavior, intestinal SAA1 and SAA2 production, and hippocampal Th17 cell accumulation. Patients with current major depressive disorder exhibited increased fecal IL-17A, SAA and SFB levels. These results reveal a novel mechanism by which bacteria alter mood.

Introduction

Major depressive-disorder (MDD) is a debilitating disease with a lifetime incidence of ~20% (1). Current treatments often lack efficacy and/or take several weeks to be effective. Recent evidence has pointed towards a role of the gut microbiome composition in exacerbating psychiatric disorders, including MDD (2). The microbiota within the gut influence gut-brain communication and behavior [for review (3)]. The bidirectional communication between the gut and the brain is thought to involve neural, hormonal and immunological routes, including the sympathetic and parasympathetic arms of the autonomic nervous system and the enteric nervous systems. Dysregulation of this communication often leads to pathophysiological effects (4).

There are well-documented effects of changes in the composition of the microbiota on behavior and cognition (5). Microbiota changes have often been studied by using germ-free animals, bacterial infection, and probiotic treatments. For example, the absence of the microbiome, such as in germ-free mice, has been associated with increased susceptibility to stress, and this can be reversed by administration of *Bifidobacterium infantis* harvested from the feces of the specific pathogen-free control animals (6). Moreover, the earlier the colonization occurs, the better the outcome. De Palma and colleagues (7) showed that germ-free mice were less immobile in the tail suspension test of depression-like behavior than conventional mice, suggesting an antidepressant action of depleting microbiota in mice. Conversely, transfer of a human fecal microbiota from depressed patients to germ-free mice conferred a depressive-like behavior to the mice compared to mice receiving microbiota from healthy patients, showing that dysbiosis of the gut microbiome can promote depressive-like behaviors in mice (8).

The microbiota can be influenced by many factors, including diet, exercise, and infection, and a major factor that is relevant for depression is stress (9–11). Substantial evidence

demonstrates that activation of the hypothalamus-pituitary-adrenal (HPA) axis influences the composition of the gut microbiota. Maternal separation in rhesus monkeys, which increases HPA axis activity, resulted in a reduction of the *Lactobacilli* Gram-negative bacteria (12, 13), and early-life stress in rats or chronic restraint stress in adult rodents also changed the composition of the gut microbiota (14), in conjunction with increasing pro-inflammatory cytokines and chemokines (15). Chronic restraint stress also increases the permeability of the gut, leading to the disruption of the gut barrier (leaky gut) and the release of lipopolysaccharides into the circulation (16, 17). This, however, can be reversed by probiotic agents (13, 17, 18). MDD patients exhibit significant changes in the relative abundance of *Firmicutes*, *Actinobacteria* and *Bacteroidetes* compared to healthy individuals (8) and administration of the probiotic *Lactibacillus farciminis* prevented the gut leakage and the activation of the HPA axis (19, 20), confirming the critical role of the microbiota in the gut-brain dialogue and the potential beneficial role of probiotic treatments in MDD (21). A variety of mechanisms of action of probiotics have been proposed, such as 1) downregulating the HPA axis, which is often overactive in MDD patients (22), 2) promoting biosynthesis of GABA, levels of which are reduced in MDD patients (23), and 3) increasing the production of tryptophan and therefore the serotonin level (24). Altogether, gut dysbiosis is often evident in MDD patients and in mice exhibiting depressive-like behaviors (8, 25–29) and for review (30), suggesting a dysregulation of the brain-gut axis in depression. However, molecular mediators of the effects of the microbiota in depressive-like behaviors remain to be identified.

Besides regulating brain responses to stress and behavior, the microbiota also dramatically regulates the immune system (31, 32). Thus, recent studies have shown the importance of host-specific species such as the *Segmented filamentous bacteria* (SFB), also called *Candidatus arthromitus*. SFB is a non-culturable spore-forming Gram-positive bacterium that is most closely related to the genus *Clostridium*, and colonizes the intestines of numerous species, including humans (33). Mouse gut colonization by SFB is required for the maturation of the innate and the adaptive immune systems (34, 35). This is relevant for depression because critical roles of the innate and adaptive immune systems have been shown to regulate the susceptibility to depression both in humans and mice (36–38). T cells express the surface marker, CD4, that is used to identify them as CD4⁺, and one type of T cells, the T helper (Th) 17 cells that produce the signature cytokine IL-17A are known to be toxic to the CNS in autoimmune diseases (39). We recently found that Th17 cells were sufficient to increase susceptibility to depressive-like behaviors in mice (40). Th17 cells are only present in the lamina propria of the small intestines in healthy mice (34), whereas they are pathogenic when they infiltrate the CNS, such as in multiple sclerosis (41). Thus, it is particularly relevant that Th17 cells are highly up-regulated by SFB (34), (42). Without SFB, Th17 cells are absent in the mouse small intestinal *lamina propria* where they are usually abundant in normal healthy rodents in the presence of commensal microbiota (34). Introduction of SFB in mice that are deficient in Th17 cells induces the production of Th17 cells (34).

Here, we tested if changes in the microbiome modulate depressive-like behaviors, and found that mice deficient in SFB are resilient to the induction of depressive-like behaviors, and that introduction of SFB in SFB-deficient mice reestablished susceptibility to depressive-like

behaviors. Bacteria communicate, detect, and respond to cell population density by releasing quorum sensing molecules (43). SFB produce the quorum sensing molecule autoinducer-2 (AI-2), a furanosyl borate diester or tetrahydroxy furan (IUPAC name: 3a*S*,6*S*,6a*R*)-2,2,6,6a-tetrahydroxy-3a-methyltetrahydrofuro[3,2-*d*][1,3,2]dioxaborolan-2-uide), and administration of AI-2 induced a host response, comprising SAA1 and SAA2 production, and promoted depressive-like behaviors in a Th17 cell-dependent manner. These experiments identified a SFB/AI-2/Th17 cell circuit as contributing to the increased susceptibility to depressive-like behaviors, uncovering the previously unknown role of bacterial AI-2 in modulating behavior.

Material and methods

Mice

6–12 week old wild-type C57BL/6, *Rorc*(γ T)^{+/GFP}, *Rag2*^{-/-} or 7B8 (Tg(Tcra,Tcrb)2Litt/J, strain 027230), IL17A-IRES-GFP-KI (strain 018472) male mice were used. C57BL/6 mice were either bred in the University of Miami animal facility or purchased from Taconic Biosciences (TAC) or Jackson Laboratory (JAX). The *Rorc*(γ T)^{+/GFP} mice (strain 007572, (44)) were obtained by crossing *Rorc*(γ T)^{+/GFP} x *Rorc*(γ T)^{+/GFP}, to produce 50% *Rorc*(γ T)^{+/GFP}, 25% wild-type, and 25% *Rorc*(γ T)^{GFP/GFP} mice, and littermates were used. *Rorc*(γ T)^{GFP/GFP} mice were not used because their locomotor activity is altered, compromising the interpretation of the behavioural testing. Mice were housed in light and temperature controlled rooms and treated in accordance with NIH and the University of Miami Institutional Animal Care and Use Committee regulations.

Treatments

Mice were injected intraperitoneally with 5 nmoles of AI-2 (Omm Scientific, vehicle is saline), sc-AHL (C6) (Sigma, vehicle is saline), or oleic acid (Sigma, vehicle is saline with 5% DMSO, 5% Tween80), or 5 μ g of mouse recombinant SAA2 (MyBioSource, vehicle is saline) as indicated within each experiment.

Behavioral assessments

-Learned helplessness: Learned helplessness was measured using a standard learned helplessness paradigm or a modified reduced intensity inescapable foot shock protocol, as described previously (40, 45). The reduced intensity paradigm was used so wild-type C57BL/6 mice did not develop learned helplessness, allowing measurements of increased susceptibility to learned helplessness. Briefly, mice were placed in one side of a Gemini Avoidance system shuttle box (Med Associates, St Albans, VT, USA) with the gate between chambers closed. For standard learned helplessness, 180 inescapable foot shocks were delivered at an amplitude of 0.3 mA, a duration of 6–10 s per shock, and a randomized inter-shock interval of 5–45 s (40). In a modified inescapable shock protocol, referred to as the reduced intensity learned helplessness protocol, mice were given 180 foot shocks with amplitude of 0.3 mA and a 2–6 s shock duration, and a randomized inter-shock interval of 5–45 s (45). Twenty-four hours after the inescapable foot shocks, mice were returned to the shuttle box and the number of escape from 30 escape trials was recorded. Each trial uses a 0.3 mA foot shock for a maximum duration of 24 s. The door of the chamber opens at the beginning of

the foot shock administration to allow the mouse to escape. Trials in which the mouse did not escape within the 24 s time limit were counted as escape failures. Mice with greater than 15 escape failures were defined as learned helpless. For the long-term paradigm, mice subjected to the standard learned helplessness were retested with escapable foot shocks once a week during a maximum of 4 weeks (46).

-Tail Suspension Test: For the tail suspension test (TST) mice were suspended by the tail on an automated TST cubicle (33 × 31.75 × 33 cm; Med Associates, St Albans, VT, USA) for a period of 6 minutes and the immobile time was analyzed for the last 4 minutes, using Med Associates software.

-Open Field: The locomotor activity in an open field activity was measured as previously described (40). Briefly, mice were placed in a Plexiglas open field (San Diego Instrument) outfitted with photobeam detectors under soft overhead lighting, and activity was monitored during 30 min using activity monitoring software (San Diego Instrument).

-Social Interactions: For the three-chambered social interaction test (40), the apparatus was a rectangular, transparent, Plexiglas box divided by Plexiglas walls into three equal-sized connected chambers with an empty wire enclosure in the two end chambers. The day prior to testing, the test mice were habituated individually by being allowed to freely explore the entire apparatus for 20 min and, separately, an unfamiliar, conspecific and same-sex stimulus mouse was habituated for 20 min into the wire enclosure in one of the chambers. On the day of the test, the test mouse was placed in the center of the middle chamber and allowed to freely explore the entire apparatus for 5 min. The test mouse was allowed to explore the entire apparatus for 10 min with the unfamiliar mouse placed in one of the chambers on the side of the box. Each session was videotaped and quantified for time spent in each chamber and for number of nose contacts with the stimulus mouse.

SFB colonization

The colonization by SFB was achieved by either co-housing 5–6 week old age-matched male mice *ad libitum* in sterilized cages for two weeks at a ratio of 2:3 (JAX:TAC) as previously described (47) or by gavage of 100 µL of fecal homogenates of SFB monocolonized mice in water (34). SFB colonization after two weeks was confirmed by qPCR. Control JAX mice were gavaged with homogenates of their own feces in water.

SFB qPCR

Genomic DNA was purified from stools using the Quick-DNA Fecal/Soil Microbe Miniprep (Zymo Research) according to the manufacturer's instructions. SFB gene expression (SFB primers: 5'-ACGCTACATCGTCTTATCTTCCCGC-3' and 5'-TCCCCAAGACCAAGTTCACG-3') was assessed by SYBR qPCR in a Jena Analytika instrument and the results were quantified by the 2^{-C_t} method. Values were normalized to *Eubacteria* (EUB primers: 5'-ACTCCTACGGGAGGCAGCAGT-3' and 5'-ATTACCGCGGCTGCTGGC-3') for each sample.

QSM measurement

Fecal homogenates were incubated with bacteria expressing the plasmids pSB406 (sc-AHL), pSB1075 (lc-AHL) or MM32 (AI-2). The same amount of feces weight was used. Briefly, for measuring AHLs, *E. coli* DH5a bacteria expressing pSB1075 or pSB406 were grown in LB containing 100 µg/mL ampicillin at 37°C with orbital shaking, whereas for measuring AI-2, *V. Harveyi* bacteria were grown overnight in M9 medium containing 30 µg/mL kanamycin, at 30°C with orbital shaking (250 rpm). Stools were homogenized in water and diluted 1:1600 (AHL) or 1:750 (AI-2), and incubated for 2 h (AHLs) or 2.5 h (AI-2) with these bacteria at 37°C (AHL) or 30°C (AI-2), with orbital shaking at 175 rpm.

Bioluminescence was assessed using a standard curve with commercial AHL or AI-2 (Sigma or Omm Scientific). The induced luminescence intensity was measured using a microplate reader (BMG Labtech Polar Star Optima microplate luminometer, Ortenberg, Germany). All luminescent intensities were reported as the average of a minimum of three replicates that are blank subtracted and are expressed in relative light units (RLU).

Microbiome sequencing:

V4 16S RNA sequencing was performed by Second Genome (South San Francisco, CA). Briefly nucleic acid isolation was achieved with the MoBio PowerMag® Microbiome kit (Carlsbad, CA) according to the manufacturer's instructions and optimized for high-throughput processing. All samples were quantified via the Qubit® Quant-iT dsDNA High Sensitivity Kit (Invitrogen, Life Technologies, Grand Island, NY). For the library preparation, each sample was PCR amplified with two differently bar coded V4 fusion primers and concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by qPCR. Samples were paired-end sequenced using a MiSeq instrument (Illumina), according to the manufacturer's instruction. Reads were merged using USEARCH and the resulting sequences were compared to an in-house strains database using USEARCH (usearch_global). All sequences hitting a unique strain with an identity 99% were assigned a strain Operation Taxonomic Unit (OTU). To ensure specificity of the strain hits, a difference of 0.25% between the identity of the best hit and the second best hit was required. For each strain OTU, one of the matching reads was selected as representative and all sequences were mapped by USEARCH (usearch_global) against the strain OTU representatives to calculate strain abundances. The remaining non-strain sequences were quality filtered and dereplicated with USEARCH. Resulting unique sequences were then clustered at 97% by UPARSE and a representative consensus sequence per de novo OTU was determined. Representative OTU sequences were assigned taxonomic classification via mothur's bayesian classifier, trained against the Greengenes reference database of 16S rRNA gene sequences clustered at 99%. All profiles are inter-compared in a pair-wise fashion to determine a dissimilarity score and stored in a distance dissimilarity matrix, using the Bray-Curtis dissimilarity. The binary dissimilarity values were calculated with the Jaccard index and represented using dendrograms and analyzed using Permutational Analysis of Variance (PERMANOVA). Univariate differential abundance of OTUs was tested using a negative binomial noise model for the overdispersion and Poisson process intrinsic to this data, as implemented in the DESeq2 package (48), and described for microbiome applications in (49). DESeq q-values were corrected using the Benjamini-Hochberg procedure. All the data were deposited in SRA, Bioproject PRJNA498103.

Antibiotics treatment

Specific-pathogen free (SPF) mice were gavaged with a solution of neomycin (100 mg/kg), metronidazole (100 mg/kg) and vancomycin (50 mg/kg) twice daily for 7 days before starting the injections of AI-2, and antibiotic treatments were continued until the behavioral assessments were finished to avoid bacterial recolonization, using dosages typically greater than those used for clinical therapeutic treatment. Ampicillin (1 mg/mL) was also provided *ad libitum* in drinking water. These conditions produced germ-free like phenotype {Reikvam, 2011 #1576}. Bacterial depletion was evaluated by *Eubacteria* 16S qPCR. The level of *Eubacteria* was 99.9% depleted (Suppl Fig 1F), confirming depletion of the microbiota with the antibiotic regimen.

Adoptive transfer

CD4⁺ cells were isolated as described previously³³. ~1×10⁶ undifferentiated CD4 cells were injected i.v. in 200µL PBS by tail vein 48 h before behavioral testing or as indicated in suppl fig 2H-I. Depending on the number of mice to be injected, cells transferred were pooled from 1–2 donors.

SAA, IL-22 qRT-PCR

The most distal part of the small intestine was dissected and rinsed and Peyer's patches were removed. RNA was extracted with TRIzol Reagent (Life Technologies) and cDNA was synthesized with ImProm-IITM Reverse Transcriptase and random primers (Promega). SAA1, SAA2, SAA3 (34) and IL-22 expression was measured by SYBR green RT-qPCR in a Jena Analytika instrument and the results were quantified by the 2^{-Ct} method. Primers used: SAA1: 5'-CATTTGTTACACGAGGCTTTCC -3' and 5'- GTTTTTCCAGTTAGCTTC CTTTCATGT -3'; SAA2: 5'- TGTGTATCCCAAG GTTTCAGA -3' and 5'- TTATTACCCTCTCCTCCTCAAGCA -3'; SAA3: 5'- CGCAGCACGAGCAGGAT -3' and 5'- CCAGGATCAA GATGCAAAGAATG -3'; IL-22: 5'- AGAACGTCTTCCAGGGTGAA -3' and 5'- TCCGAGGAGTCAGTGCTAAA -3'. Values were normalized to GAPDH (Primers: 5'- AGGTCGGTGTGAACGGATTTG -3' and 5'- TGTAGACCATGTAGTTGAGGTCA -3').

Flow cytometry

Immediately after learned helplessness, mice were anesthetized, spleens were recovered, and mice were transcardially perfused with PBS as previously described (40, 50). Briefly, the hippocampi were dissected excluding meninges and choroid plexus, passed through a 70 µm cell strainer (BD Bioscience) and the cell suspension was mixed (vol/vol) to obtain a 30% Percoll/R1 medium [RPMI 1640 medium (Corning) supplemented with 1% FBS (Gibco), 100 IU/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), 1 × non-essential amino acids (Gibco), 1 µM sodium pyruvate (Gibco), 2.5 µM β-mercaptoethanol (Sigma) and 2 mM L-glutamine (Gibco)]. The cellular suspension was overlaid on 70% Percoll/R1 medium in a centrifuge tube, and centrifuged at 2000 rpm for 20 min without using the brake. The cells at the interface of the 30/70% Percoll gradient were recovered, washed once, resuspended in R10 media and stimulated for 4 h with phorbol myristate acetate (PMA, 50 ng/mL; Sigma) and ionomycin (750 ng/mL; Sigma) in the presence of Protein

Transport Inhibitor Cocktail (eBioscience) at the recommended concentrations. Standard intracellular cytokine staining was carried out as described³³ using the Staining Intracellular Antigens for Flow Cytometry Protocol (eBioscience). Cells were first stained extracellularly with BV480-conjugated anti-CD4 (BD Bioscience), then fixed and permeabilized with permeabilization solution (eBioscience) and finally intracellularly stained with BV650-conjugated anti-IFN- γ (BD Bioscience), phycoerythrin-conjugated anti-IL-17A (ebioscience) and BV421-conjugated anti-FoxP3 (BD Bioscience). Samples were acquired on a FACS CELESTA (BD Bioscience) and data were analyzed with FlowJo software (Tree Star, Inc.).

Immunohistochemistry

Immediately after learned helplessness escapable-shock testing, mice were anesthetized, spleens were recovered, and mice were transcardially perfused with PBS as described above. Brains were fixed with 4% PFA, post-fixed, cryo-protected in OCD, snap-frozen and stored at -80°C . Twenty μm sections were stained for GFP (anti-GFP Chicken, #1020, Aves labs Inc) and Dapi and imaged on a EVOS FL fluorescent microscope (Life Technologies).

Gut permeability

To measure gut permeability (modified from (51)), mice were gavaged with FITC-Dextran (Sigma; 600 mg/kg in 100 μL of PBS), 4 h before sacrifice. Mice were anesthetized and blood was collected at the time of sacrifice via hepatic vein and spun down to collect serum. Fluorescence was measured at 490 nm (excitation) and 530 nm (emission) in serum samples diluted 1:1 with PBS.

Cytokine measurements

Serum cytokines were measured in 12.5 μL of serum using multiplexing measuring 26 cytokines and chemokines according to the manufacturer's instructions (m cytokine/chemokine, 26 Plex, cat#EPX260-26088-901, ebioscience) on a MAGPIX instrument (Luminex).

Human samples

Stool samples from 10 participants with current depressive symptoms defined as a Quick Inventory of Depressive Symptomatology (QIDS) score of 13 and 10 matched healthy controls were analyzed (demographic information in Table S1) from an ongoing, longitudinal study at the University of Texas Southwestern Medical Center. Samples were collected by participants at home, frozen upon collection, transported frozen back to the center and then stored at -80°C until analysis.

80% of the depressed sample had primary diagnosis of Major Depressive Disorder (MDD) and the remaining psychiatric sample had Bipolar Disorder diagnoses. Psychiatric state at the time of sample collection assessed by the Longitudinal Interview Follow-Up Evaluation (LIFE) (73) confirmed the incidence of a full depressive episode or an episode in partial remission for all. In addition, the majority of the psychiatric sample had marked or fully expressed Generalized Anxiety Disorder (GAD) and/or other non-GAD anxiety disorder, and two participants experienced mania or hypomania within 2 weeks of sample collection.

One participant's LIFE data was not available. The healthy control samples had no diagnosed psychiatric disorders. The study was approved by the University of Texas Southwestern Institutional Review Board. Informed consent was obtained for all participants.

Human IL-17A and SAA measurement

Stool samples were homogenized (100 mg/mL) in PBS supplemented with 1 µg/mL leupeptin, pepstatin and 1 mM phenylmethylsulfonyl fluoride, sonicated and centrifuged 15 min at 10,000 g at 4°C. IL-17A and SAA were measured in 25 µL of stool homogenates using human IL-17A Simplex and SAA Simplex Procarta multiplex assays (cat#EXP01A-12136-901, EXP01A-12017-901, Thermo Fisher Scientific) on a MAGPIX instrument (Luminex). Samples were run on 1 plate, blind to treatment. Assays were checked for quality control to fit the standard curves. Two samples (1 HC and 1 MDD, who had a primary Bipolar diagnosis) were not used for IL-17A and SAA measures because of the nature of the stools (diarrhea), for which 50 mg of actual stool could not be sampled to get protein concentrations consistent with the other samples. This issue did not affect QSM measurements because they are chemicals measured on the basis of tissue weight rather than protein concentration. Points were excluded if the values deviated more than 3 SD from the mean and were considered statistically outlier values. For all analyses, any samples that were under the detection limit were excluded. The data were analyzed using SPSS Version 24.

Human SFB and QSM measurement

For SFB measurements, genomic DNA was extracted using the Quick-DNA Fecal/Soil Microbe Miniprep (Zymo Research) according to the manufacturer's instructions, and amplified by PCR as described above. Points were excluded if the values deviated more than 3 SD from the mean and were considered outlier values. For the QSM measurements, similar approaches as for mouse were used as described above.

Statistical analysis

Data are represented as mean ± SEM. Statistical significance was analyzed with a one-way analysis of variance (ANOVA) for multiple comparisons with Bonferroni post-hoc test or with Student's *t*-test or Mann-Whitney using Prism software when appropriate. Pearson correlation were performed on SPSS version 24. **p*<0.05 was considered significant. All statistical tests were two-sided.

Results

To determine if the composition of the gut microbiome can affect sensitivity to the foot shock-induced learned helplessness model of depression (Fig 1A), we tested the sensitivity to the induction of learned helplessness of mice obtained from The Jackson Laboratory (JAX) or Taconic Farms (TAC), which have different microbiomes(34). We found that only 21% of JAX mice, but 67% of TAC mice, exhibited learned helplessness (Fig 1B), showing that TAC mice were significantly more susceptible to the induction of learned helplessness than JAX mice. We next tested if co-housing JAX and TAC mice, which allows sharing of microbiota between co-housed mice, changed their susceptibility to learned helplessness.

After co-habitation for 15 days, 56% of JAX mice and 53% of TAC mice exhibited learned helplessness (Fig 1B). This suggests that JAX mice that acquire the microbiota of TAC mice (34) display increased susceptibility to learned helplessness compared with naïve JAX mice, averaging 18 failures out of the 30 escape trials compared to 8 failures without co-housing, whereas the susceptibility of TAC mice remained essentially unchanged by co-habitation. This raises the possibility that bacteria in the TAC mice microbiota increases sensitivity to learned helplessness, and/or the microbiota of JAX mice provides resilience to learned helplessness.

To identify bacteria that may modulate learned helplessness, TAC mice were retested every week to identify mice that maintain learned helplessness for a long period of time (Fig 1 C). For this, we used the paradigm of learned helplessness we recently described (40), in which ~20% of the mice keep exhibiting learned helplessness after 3–9 weeks of retesting (Fig 1C). This differs from the behavior of most mice, as ~80% of mice spontaneously recover from learned helplessness within 3 weeks, demonstrating a difference in 20% of mice that blocks recovery from this depression-like behavior. We performed V4 16S rRNA sequencing to identify the bacteria that are different in mouse stools immediately after exposure to escapable foot shocks (to differentiate mice that are either susceptible or resilient to learned helplessness) and in mice that maintain learned helplessness for 4 weeks (Fig 1D-E). We ensured a complete microbiome profile was captured because the number of sequences per sample ranged from 111,769 to 162,600 filtered reads. We found no change in the alpha diversity metric, and the average number of OTUs per samples and per group ranged from 342–368, with an average Shannon diversity index per group range of 2.77–3.47. There were no differences among groups at the level of phyla. However, at the family level, *Erysipelotrichaceae* was lower in mice 4 weeks after receiving inescapable foot shocks whether they exhibited learned helplessness (2.26%) or did not display learned helplessness (2.89%) compared to mice that did not receive foot shocks (mean 22.7%) (Fig 1D). Thus, the stress of the learned helplessness paradigm was sufficient to cause this large difference in the microbiota independently of the behavioral outcome. There were 6 significantly different operational taxonomic units (OTU) detected out of 497 examined in mice displaying learned helplessness for 4 weeks compared to mice that underwent the learned helplessness paradigm but never displayed learned helplessness. *Akkermansia muciniphila* (1 OTU) and *Mucispirillum schaedleri* (2 OTU) were enriched in non-learned helplessness samples (Fig 1G), whereas *Segmented filamentous bacteria* (SFB) (1 OTU) and 2 unclassified OTUs from the order *Clostridiales* were enriched in mice displaying learned helplessness for 4 weeks (Fig 1F). Thus, the microbiome is significantly different in mice that are resilient to learned helplessness and mice that are susceptible and maintain prolonged learned helplessness.

The elevated SFB level in mice that exhibited prolonged learned helplessness compared with resilient mice raised the possibility that SFB may promote learned helplessness. Interestingly, JAX mice, which are resistant to learned helplessness, are known to be deficient in SFB (Fig 1H, (34)), and we found that JAX mice co-housed with TAC mice express fecal SFB similar to TAC mice (Fig 1H) as well as exhibiting similar susceptibility to learned helplessness (Fig 1B). We tested if SFB is sufficient to increase susceptibility to learned helplessness by gavaging JAX mice with SFB monocolonized feces, and subjecting them to learned helplessness. After the introduction of SFB, 64% of JAX mice exhibited

learned helplessness (compared with 21% in control JAX mice shown in Fig 1B), within an average 20 failures out of 30 trials (Fig 1B). These results indicate that the presence of SFB in the microbiota promotes susceptibility to learned helplessness, but does not rule out the likelihood that other microbiota components also are modulatory, such as *Akkermansia muciniphila* and *Mucispirillum schaedleri*.

To identify a mechanism by which bacteria in the microbiome may influence depression-like behaviors, we examined quorum-sensing molecules (QSM). QSMs are uniquely secreted by bacteria in response to changes in population density to coordinate several bacterial behaviors, including biofilm formation, swarming, virulence gene expression, as well as modulating antibiotic resistance (52, 53). We hypothesized that QSMs, which would reflect overall changes in bacteria species in the gut, might contribute to microbiome-modulated susceptibility to learned helplessness. We analyzed 3 major QSMs in the feces of the JAX, TAC and co-housed mice: autoinducer-2 (AI-2), short chain AHL (sc-AHL), and long chain AHL (lc-AHL). The fecal AI-2 level was significantly increased in TAC mice or JAX mice co-housed with TAC mice compared to JAX mice alone (Fig 2A). The abundance of sc-AHL and lc-AHL were similar among all groups (Fig 2B-C). Reintroduction of SFB in JAX mice was sufficient to significantly increase AI-2 levels but not sc-AHL or lc-AHL (Fig 2A-C). We confirmed that AI-2 can be produced by SFB, as the fecal AI-2 level was increased in germ-free mice monocolonized with SFB compared to TAC mice (Fig 2D), whereas sc-AHL or lc-AHL levels were similar in these two groups (Fig 2E-F), suggesting that AI-2, sc-AHL and lc-AHL were all produced by SFB, but that AI-2 might be preferentially expressed in SFB monocolonized mice (Fig 2D). Germ-free mice do not produce AI-2, sc-AHL or lc-AHL (Fig 2D-F). We also determined if foot shock stress used for inducing learned helplessness induced AI-2 levels. TAC mice subjected to the 48 h paradigm of learned helplessness had a higher level of AI-2 than TAC mice that did not receive foot shocks (Fig 2G), whereas the levels of sc-AHL and lc-AHL remained unchanged between these two groups (Fig 2H-I), indicating that the stress of foot shocks was sufficient to increase AI-2 levels as well as to induce learned helplessness, suggesting that bacteria might adapt their behavior after the induction of stress (Fig 2J).

Since AI-2 was associated with increased susceptibility to learned helplessness in JAX mice, we tested if AI-2 is sufficient to promote depressive-like behaviors (Fig 3A) by daily treatments of AI-2 for 3 days prior to testing mice in the reduced intensity paradigm of learned helplessness. With this paradigm, most mice do not develop learned helplessness (40, 45). AI-2 administration, but not sc-AHL, was sufficient to increase susceptibility to learned helplessness in both TAC mice (Fig 3B) and JAX mice (Suppl Fig 1A). Similarly, administration of AI-2 increased immobile time in the tail suspension test (Fig 3C), often interpreted as an indication of increased despair or depression. In a measure of sociability, which is frequently impaired in depression and models of depression, administration of AI-2 reduced the number of nose contacts with an unfamiliar mouse (Fig 3D), without interfering with the preference for the CH1 chamber containing the new mouse (Fig 3E). AI-2 administration did not alter locomotor activity in a novel open field (suppl Fig 1C-E). To determine if bacteria were required for AI-2 pro-depressant effects, we treated TAC mice and JAX mice with antibiotics to deplete the microbiota, and measured behaviors. Antibiotics treatment depleted significantly the microbiota as shown by the reduced

Eubacteria levels (Suppl Fig 1F) and prevented AI-2 from promoting depressive-like behaviors (Suppl Fig 1G-H), suggesting that AI-2 acts on bacteria rather than the host to promote depressive-like behaviors. Taken together, these findings demonstrate that AI-2 administration increased displays of multiple depressive-like behaviors that are microbiome-dependent.

SFB is well-established to promote production of Th17 cells(34), which promote depressive-like behaviors(40, 45), and Th17 cells accumulate in the hippocampus of mice exhibiting learned helplessness (45) and are localized in the brain parenchyma (Suppl Fig 2A). Introduction of SFB in JAX mice increased the accumulation of hippocampal Th17 cells after learned helplessness (Suppl Fig 2B-D), whereas there was no difference in splenic Th17 cells (data not shown). We next investigated whether commensal-antigen specific CD4⁺ cells are sufficient to promote learned helplessness in SFB⁺Rag2^{-/-} mice. CD4⁺ cells from mice expressing a transgenic T cell receptor (TCR) specific for SFB-encoded antigen (7B8) or CD4⁺ cells from a littermate wild-type mice were adoptively transferred to Rag2^{-/-} mice, and subjected to the reduced intensity paradigm of learned helplessness. We found that 75% of Rag2^{-/-} mice receiving 7B8 CD4⁺ cells exhibited learned helplessness with an average of 23 escape failures, whereas none of the Rag2^{-/-} mice receiving wild-type CD4⁺ cells exhibited learned helplessness, averaging 0.5 escape failures (Suppl Fig 2E). Consistently, hippocampal Th17 cells, but not Th1 cells, were significantly increased in Rag2^{-/-} mice receiving 7B8 CD4⁺ cells and exhibiting learned helplessness, whereas receiving 7B8 CD4⁺ cells without receiving foot shocks was not sufficient to increase hippocampal Th17 cells (Suppl Fig 2F-G). Thus, the presence of SFB in the microbiota represents one factor that modulates Th17 cell trafficking during depression.

Since SFB promotes learned helplessness, hippocampal Th17 cell accumulation during learned helplessness, and produces AI-2, we tested if AI-2 administration increases Th17 cells in the hippocampus. After administration of AI-2, mice were subjected to the reduced intensity paradigm of learned helplessness because this paradigm does not increase hippocampal Th17 cell accumulation(50), allowing for studies of factors that promote this outcome. Th17 cells were increased 3-fold in the hippocampus of mice receiving AI-2 compared to vehicle- or sc-AHL-treated mice (Fig 3F), whereas AI-2 had no effect in the absence of foot shocks (suppl Fig 2H), or in mice treated with antibiotics to deplete the microbiota (Suppl Fig 2I), demonstrating that AI-2 promotion of Th17 cell accumulation in the hippocampus is stress (foot shocks)- and microbiota-dependent. Furthermore, even though AI-2 did not promote Th17 cell differentiation *in vitro* (data not shown), AI-2 administration promoted Th17 cell differentiation *in vivo*, as indicated by the finding that AI-2 administration increased the percent of GFP⁺CD4⁺ cells both in the spleen (Fig 3G) and the hippocampus (Fig 3H) after learned helplessness in RORc(γ T)^{+/GFP} mice, where a GFP reporter cDNA was knocked-in at the site of initiation of *ROR γ T* translation, leading to an important decrease in Th17 cells (40, 44, 50), indicating that AI-2 was sufficient to induce Th17 cells after shocks. Since AI-2 promotes both Th17 cell differentiation and depressive-like behaviors, we tested if Th17 cells mediate the depression-promoting effect of AI-2 by using RORc(γ T)^{+/GFP} mice. RORc(γ T)^{+/GFP} mice are resistant to the induction of learned helplessness but adoptive transfer of Th17 cells is sufficient to resensitize RORc(γ T)^{+/GFP} mice to learned helplessness⁴¹. The pro-depressive effects of AI-2 administration

were abolished in RORc(γ T)^{+/GFP} mice (Fig 3B-D), and this was independent of SFB, AI-2, sc-AHL, lc-AHL or SAA levels (Suppl Fig 3), or locomotor activity (Suppl Fig 1C), which were similar in untreated wild-type and RORc(γ T)^{+/GFP} mice. This indicates that Th17 cells are required to mediate AI-2 induced depressive-like behaviors.

Because Th17 cells primarily reside in the *lamina propria* of the small intestines in healthy mice(34), and SFB promotes Th17 cell differentiation in the small intestines via the SAA1–2/IL-22 pathway (34, 35, 54), we tested if intestinal SAA1, SAA2, SAA3 and IL-22 levels were affected by administration of AI-2 in TAC mice after stress (Fig 4A). We found that AI-2 administration increased the intestinal expression of SAA1 and SAA2 (Fig 4B-C) but did not change the intestinal expression of SAA3 and IL-22 (Fig 4D-E) and that antibiotics treatment abolished this effect (Suppl Fig 4A-B). Furthermore, mice exhibiting prolonged learned helplessness (4 weeks), also exhibited higher intestinal levels of SAA1 and SAA2 (Fig 4F-G), but not SAA3 and IL-22 (Fig 4H-I), compared to mice that did not exhibit learned helplessness or non-shocked mice. Consistent with the report of Ivanov *et al.* (34), SFB administration increased intestinal SAA1, SAA2, SAA3 and IL-22 levels (Suppl Fig 4C-F). Furthermore, there were reductions of SAA1 and SAA2 levels after induction of learned helplessness in RORc(γ T)^{+/GFP} mice compared to wild-type mice (Suppl Fig 5A-D) suggesting that RORc(γ T)^{+/GFP} mice that are resilient to learned helplessness, also exhibit lower levels of SAA1 and SAA2. Furthermore, injection of recombinant SAA2 increased susceptibility to learned helplessness by 50% (Suppl Fig 5E). Taken together these findings suggest the increase of intestinal SAA1 or SAA2 levels after AI-2 administration might be sufficient to increase susceptibility to learned helplessness.

Since SAA promotes intestinal cytokine production, serum cytokine and chemokine levels were measured from 3 to 48 h after AI-2 treatment to determine if inflammation might be contributing to the induction of Th17 cells and depressive-like behaviors after AI-2 treatment. The prototypic proinflammatory cytokines known to induce depressive-like behavior IL-1, IL-6 and TNF (36, 38) were not affected by AI-2 treatment (Suppl Fig 6). However, there were increased serum levels of IL-2, IL-13, IL-17A, G-CSF and decreased levels of IL-4, IL-5, IL-12, GM-CSF, CXCL1, CCL2 and CCL5 after AI-2 treatment (Suppl Fig 6) suggesting a remodeling of the inflammatory landscape by AI-2.

A screen of natural compounds identified oleic acid as a potential AI-2 inhibitor(55). We tested if oleic acid administration *in vivo* prevented the production of AI-2 and the induction of learned helplessness. Oleic acid administration reduced fecal levels of AI-2, but not sc-AHL or lc-AHL in mice subjected to the learned helplessness paradigm (Fig 5A-C), confirming the ability of oleic acid to inhibit AI-2 production *in vivo*. Oleic acid reduced the susceptibility to learned helplessness of TAC mice, from an average of 24 failures/30 trials to 13 failures, and reduces the percent of learned helpless mice from 78% to 40%, suggesting an antidepressant action of oleic acid (Fig 5D). The co-administration of AI-2 with oleic acid abolished this antidepressant effect, resulting in an average of 27 failures/30 trials (Fig 5D), demonstrating the requirement of AI-2 in the antidepressant effect of oleic acid. As expected, levels of factors downstream of AI-2, SAA1 and SAA2, but not SAA3 or IL-22 levels in the intestine were reduced by oleic acid administration (Fig 5E-H) after learned helplessness. However, only SAA2 levels were restored to vehicle levels after AI-2

co-administration, suggesting that SAA2 mediates AI-2-dependent depressive-like behaviors. Furthermore, the percent of Th17 cells was also reduced in oleic acid-treated mice, whereas Th1 and Tregs were not affected (Fig 5I-K). This reduction of Th17 cells was not associated with changes in gut permeability (Suppl fig 7). Altogether, these data provide a new circuit involving SFB/AI-2/SAA2/Th17 cells in promoting depressive-like behaviors after stress in mice (Fig 5L).

To determine if the findings in mice are relevant for humans, we analyzed the levels of IL-17A, SAA, AI-2 and SFB in the stools of 10 patients with a current primary diagnosis of major depressive disorder (MDD) and 10 healthy matched subjects. We found that the IL-17A level was ~8 fold increased in patients with current MDD compared to healthy subjects (Fig 6A), and SAA and SFB levels were increased ~2 fold (Fig 6B, C), whereas AI-2, sc-AHL, or lc-AHL levels were not different between groups (Fig 6 D-F). Furthermore, regression analyses showed that AI-2 levels were predicted by the presence of fecal SFB in patients with current MDD and healthy subjects; that is, SFB levels predicted AI-2 levels ($p = 0.019$, $r = 0.494$, Table 1). Similarly, SFB levels predicted SAA levels ($p = 0.004$, $r = 0.336$), SAA levels predicted IL-17A levels ($p = 0.011$, $r = 0.288$) and IL-17A levels predicted QIDS scores ($p = 0.013$, $r = 0.245$) (Table 1). No interaction of SFB with sc-AHL or lc-AHL was observed ($p = 0.335$, and $p = 0.217$). These data suggest that patients with current MDD have a dysregulation of the intestinal SFB/AI-2/SAA/IL-17A circuit.

Discussion

We uncovered a novel mechanistic circuit linking the gut microbiome and bacterial products to the brain axis that regulates depressive-like behaviors in mice. SFB in the gut increases the bacterial QSM AI-2 and the levels of SAA1 and SAA2, and these increase the production of Th17 cells that promote depressive-like behaviors ((54), fig 2–4). Furthermore, administration of either SFB or AI-2 is sufficient to increase susceptibility to depressive-like behaviors of SPF mice with an intact microbiome. We also found that disrupting this signaling pathway by blocking AI-2 with oleic acid provides antidepressant properties. These findings reveal a signaling mechanism by which changes in bacterial products influence mood-relevant behavior.

As has previously been reported, microbiota composition was found to influence behaviors (2, 8, 56). We identified SFB, a spore forming bacteria belonging to the *Firmicutes* phylum and closely related to the *Clostridium* family, as a candidate bacteria that influences mood-relevant behaviors. This extends previously published reports that *Firmicutes* are modulated in mice after stress exposure (9, 25, 57). Shifts in the composition of the two predominant phyla of the gut microbiome, *Bacteroidetes* and *Firmicutes*, have been associated with several CNS conditions (e.g. autism, stress, Alzheimer's disease) (58, 59). Although the composition of the gut microbiota has been associated with depressive disorders, the mechanisms whereby gut bacteria modulate mood remains largely unknown (25, 60). We propose that induction of Th17 cells by *SFB* in the gut increases susceptibility to depressive-like behaviors. However, it is not excluded that other bacteria [such as *Akkermansia muciniphila* and *Mucispirillum schaedleri*, the latter being shown to be decreased after stress in another study (61)], are also involved in the modulation of depressive-like behaviors.

Akkermansia muciniphila is known for its anti-inflammatory properties (62), which could also modulate the inflammation associated with depressive-like behavior (36, 38). In addition to bacterial abundance, interactions between bacteria might be important to regulate behaviors, however further experiments are needed to test this hypothesis. Our evidence indicates that the bacterial quorum-sensing molecule AI-2 can be an important conduit of signals from the gut to the brain. This was particularly evident from the finding that AI-2 administration was sufficient to promote depressive-like behaviors, which also demonstrated that gut bacterial metabolites have the capacity to modulate behaviors in SPF mice. In addition, increased availability of AI-2 increases the ratio of *Firmicutes* to *Bacteroidetes*, favoring the *Firmicutes* (63), which might create a vicious circle, amplifying depressive-like behavior. AI-2 is produced by SFB bacteria and is a key link between SFB and the production of Th17 cells, one mechanism by which SFB and AI-2 can promote susceptibility to depressive-like behaviors. However, we found that AI-2 did not act directly on the host demonstrating that AI-2 mediates its effects through bacteria. Levels of AI-2 (Fig 2) but not SFB (data not shown) increased after foot shocks suggesting that either foot shocks specifically affect SFB-dependent production of AI-2 or more likely other bacteria producing AI-2 are affected by foot shocks and are responsible for the increased production of AI-2. We also found increased SFB levels in AI-2 treated JAX mice after foot shocks, suggesting that AI-2 is able to increase the population of SFB (Suppl Fig 1B), which contributes to the increased AI-2 production. But, because other bacteria also produce AI-2, this signaling circuit is not limited to SFB (64). Although AI-2 is normally produced in the lumen of the intestines, we used ip administration so further studies using intra-luminal administration would more loosely model its endogenous mode of production.

Reports have shown the presence of AI-2 and AHLs in humans, in particular in inflammatory bowel disease patients (64, 65), who are known to often experience comorbid depression(66). Consistent with this, we were also able to detect AI-2 in the stools of patients with current MDD. To our knowledge, quorum-sensing molecules have not been shown to modulate behaviors previously, but are critical to regulate biofilm formation and bacterial virulence (52, 53). Therefore, the identification of AI-2 as a modulator of depressive-like behavior represents a novel discovery that may help to clarify mechanisms by which the gut microbiome influences behavior and mood.

Widely distributed and abundant in nature, oleic acid is a monounsaturated omega-9 fatty acid that was reported to be deficient in MDD patients (67), and increased in stress-resilient rats(68), suggesting that oleic acid helps coping with stress. Oleic acid has also been associated with the maintenance of mental well-being (for review (69–71)) and treatment response to the antidepressant imipramine (72). Intake of oleic acid in women was associated with lower risk of severe depressed mood (73). Oleic acid is the predominant monounsaturated fatty acid in olive oil, and it has been proposed to contribute to the beneficial effect of olive oil (74). We confirmed that oleic acid is an AI-2 inhibitor(55) and that oleic acid exhibited antidepressant properties in the learned helplessness model of depression, which could be reversed by the addition of AI-2. This showed that the antidepressant effects of oleic acid were indeed AI-2 dependent.

Interest in the role of Th17 cells in depression has been increasing during the last several years (75). Th17 cells are usually thought of as being pathogenic in autoimmune diseases (75). Th17 cells are predominantly localized in the *lamina propria* of the small intestines in healthy mice, resulting from the activation of an IL-22-ILC3-SAA1/2 pathway induced in response to SFB, which only colonizes the ileum (54). We found that SAA1 and SAA2, but not SAA3, increase in mice exhibiting learned helplessness and in mice receiving AI-2, and decrease after oleic acid treatment, suggesting that the SAA1/2 pathway is induced after AI-2 treatment. Consistent with this, we found that SAA2 is sufficient to increase susceptibility to learned helplessness and reintroduction of AI-2 in oleic acid-treated mice only increased SAA2 but not SAA1. However, we did not find any difference in IL-22 levels after learned helplessness or AI-2 treatment, and IL-22 neutralization did not provide any antidepressant actions (data not shown), suggesting that the ILC3 cells might not be involved in promoting Th17 cell production after stress. Altogether this suggests that after stress AI-2 promotes the differentiation of Th17 cells independently of the IL-22-ILC3 pathway and that the IL-22-ILC3 pathway might only be necessary during the step of colonization by SFB to induce Th17 cells (54). Nevertheless, AI-2 promotes SAA1 and SAA2 production leading to increased Th17 cell production (54) and increased hippocampal Th17 cell accumulation. Even though Th17 cells accumulate in the hippocampus after AI-2 treatment, it remains to be determined if the Th17 cells produced in the intestines are indeed migrating to the brain. We found that AI-2 promotes Th17 cells in the brain, oleic acid reduces hippocampal Th17 cells, and SFB-specific TCR CD4⁺ cells promote depressive-like behavior in Rag2^{-/-} mice increasing hippocampal levels of Th17 cells, supporting the idea that intestinal Th17 cells migrate to the hippocampus. However further experiments are required to determine the role of hippocampal Th17 cells in promoting depressive-like behaviors, the migratory mechanisms whereby Th17 cells exit the intestines after stress and how intestinal Th17 cells infiltrate the brain. Indeed, we did not find differences in the gut permeability after AI-2 or oleic acid treatments compared to controls, whereas both AI-2 and oleic acid modulate the levels of hippocampal Th17 cells and behaviors. Therefore, it is possible that rather than changing gut permeability, and because AI-2 could not be detected in the blood (data not shown), AI-2 induces changes in Th17 cell migratory properties, such as the production of chemokine factors that attract Th17 cells outside of the gut. Consistent with this idea we found changes in serum chemokine levels after AI-2 administration, but further experiments will be needed to answer this question. It is important to note that Th17 cells were recently shown to promote maternal immune activation associated autistic traits by inducing IL-17A-dependent neurodevelopmental abnormalities in the offspring (76), suggesting that the cytokine IL-17A is sufficient to mediate some of the effects of Th17 cells in autism. The induction of Th17 cells was dependent on the presence of SFB in the ileum of the pregnant mothers (47), suggesting an important SFB/Th17 circuit in priming behaviors, even though the outcome behaviors may differ depending on the timing of induction and the location of Th17 cells.

Th17 cells have been shown to be induced by high salt diet (77) and to mediate dietary salt-induced neurovascular and cognitive impairments (78), reinforcing the idea that healthy diet, by preventing the increase of Th17 cells, enhances mental health.

Furthermore, we found fecal IL-17A levels were increased in patients with current MDD and predicted QIDS scores, reinforcing the previously found contribution of Th17 cells to MDD (79, 80). Associated with a potential intestinal Th17 cell increase in MDD, were increased fecal levels of SFB and SAA, whereas QSM levels did not change. This suggests that targeting the Th17 cell pathway might be sufficient to improve MDD. However, the sample size of our study is small and larger investigations are required.

Together, these data uncovered a novel SFB/AI-2/SAA1–2/Th17 cells pathway that promotes depressive-like behavior and reducing AI-2 might provide therapeutic benefit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Learned helpless mice exhibit higher levels of SFB.

A, Scheme of the experiment. B, JAX and TAC mice were housed with or without mice from the other source for 2 weeks, or JAX mice were gavaged with SFB or their own feces as control, and then mice were subjected to the learned helplessness paradigm. Escape failures were recorded and means±SEM of the escape failures are displayed, and the percent of mice exhibiting learned helplessness (failing to escape >15 out of the 30 trials) is shown above each group. Each symbol represents an individual mouse. Since both displayed resistance to LH, results were combined for untreated JAX mice (1/7 developed LH; average

5 escape failures) and JAX mice gavaged with their own feces (2/7 developed LH; average 8 escape failures). n=9–16, One-way ANOVA $F(4,57)=2.523$, $*p<0.05$ Bonferroni post-hoc test. C, TAC mice were subjected to learned helplessness and re-tested with only escapable shocks after 1, 2, 3 and 9 weeks and the percent of learned helpless (LH) mice was calculated after each test (n=15). (D-G) Stools were collected from mice tested in (C) after 4 weeks and V4 16S RNA gene sequencing carried out by Second Genome. D, Proportional abundance in LH mice (n=5), non-LH mice (failed to escape<15 out of 30 trials every week for 4 weeks; n=5) or mice receiving no shock (n=3). Plot shows the most abundant taxa at the Family level. E, Weighted ordination. Dimensional reduction of the Bray-Curtis distance between microbiome samples, using the PCoA ordination method. F-G, Summary of the main strains differences are shown: F, relative abundance of *Segmented Filamentous Bacteria* (SFB) compared to other OTUs in the same community is increased in learned helpless mice. G, The relative abundance of *Mucispirillum Schaedleri* and *Akkermansia Muciniphyla* compared to other OTUs in the same community is decreased in learned helpless mice. (Kruskal-Wallis rank sum test with Bonferroni correction, $*p=0.007$, n=5 mice/group compared to non-LH mice). (H) SFB levels, presented as the ratio of SFB/eubacteria were measured prior to learned helplessness induction in each group shown in (B). n=7–11, One-way ANOVA $F(4,39)=2.612$, $*p<0.05$ Bonferroni post-hoc test

Figure 2: AI-2 level increases with SFB colonization.

JAX and TAC mice were housed with or without mice from the other source for 2 weeks, or JAX mice were gavaged with SFB, and then mice were subjected to the learned helplessness paradigm. (A) AI-2 (B) sc-AHL and (C) lc-AHL levels were analyzed by luminescence in stools collected after the last foot shocks of the learned helplessness paradigm. Each symbol represents an individual mouse. Data are means±SEM. $n=4-11$ mice/group, One-way ANOVA, $F(4,34)=24.27$, $*p<0.05$, Bonferroni post-hoc test. Levels of (D) AI-2 (E) sc-AHL and (F) lc-AHL in TAC mice and in germ-free (GF) mice monocolonized or not with SFB. Each symbol represents an individual mouse. Data are means±SEM. $n=3-4$ /group, Mann-Whitney, $U=0$, $*p=0.0571$, nd, non-detectable. Levels of (G) AI-2 (H) sc-AHL and (I) lc-

AHL were measured in TAC mice subjected to learned helplessness (S) or in non-shocked TAC mice (NS). Each symbol represents an individual mouse. Data are means $\pm$ SEM. n=3–5/group, Mann-Whitney, U=0, *p<0.05. J, Scheme of the results.

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Figure 3: AI-2-mediated depressive-like behaviors are Th17 cell-dependent.

A, Scheme of the hypothesis. B-F, Wild-type or $ROR\gamma T^{+/GFP/+}$ littermate mice were injected with 5 nmoles/mouse of AI-2, sc-AHL or saline i.p. daily for 3 days. On the third day 1 hr after injection mice were subjected to (B) the reduced intensity learned helplessness paradigm and escape failures were recorded, (C) in another cohort of mice, immobile time was assessed in the tail suspension test, and (D) on the fourth day sociability was assessed with the mice used in (C) and nose contact (D) and (E) time spent in each chamber were recorded. Each symbol represents an individual mouse. Data are means $\pm$ SEM. B, n=10–12 mice/group, one-way ANOVA, $F(3,38)=5.955$, $*p<0.05$, Bonferroni's post-hoc; n=9–14

mice/group one-way ANOVA, $F(3,39)=4.224$, $*p<0.05$, Bonferroni's post-hoc (C), $n=5-11$ mice/group Mann-Whitney $U=5.5$ $*p<0.05$ (D). E, $n=4-8$ mice/group, one-way ANOVA, $F(5,25)=14.06$, $*p<0.05$, Bonferroni's post-hoc. F, Th17 cells in the hippocampus were analyzed by flow cytometry after the learned helplessness paradigm in TAC mice. Each symbol represents an individual mouse. Data are means $\pm$ SEM. $n=7-10$ mice/group, one-way ANOVA, $F(2,24)=3.56$, $*p<0.05$, Bonferroni's post-hoc test. G-H, RORc(γ T) $^{+}/GFP$ mice were injected with 5 nmoles/mouse of AI-2 or saline i.p. daily for 3 days, subjected to the reduced intensity learned helplessness paradigm on the third day and sacrificed just after the last foot shocks, and (G) splenic or (H) hippocampal GFP $^{+}$ CD4 $^{+}$ cells were analyzed by flow cytometry. Each symbol represents an individual mouse. Data are mean $\pm$ SEM. $n=4-11$ mice/group, Mann-Whitney, $U=7.5$, $*p<0.05$ (G), and $U=7.5$, $*p=0.0586$ (H).

Figure 4: Intestinal SAA1 and SAA2 levels are elevated in AI-2-treated mice.

A, Scheme of the hypothesis. B-E, TAC mice were injected i.p. with AI-2 (5 nmole/mouse) or saline daily for 3 days. 2 h after the last injections, mice were sacrificed, intestines were recovered, and RNA extracted. Expression of SAA1 (B), SAA2 (C), SAA3 (D), IL-22 (E) and GAPDH were measured by qRT-PCR. Each symbol represents an individual mouse. Mean $\pm$ SEM, n=6–8 mice/group, Mann and Whitney * p <0.05, U= 5 (B), U=8 (C). F-I, TAC mice were not shocked (NS) or subjected to learned helplessness and were retested every week for 4 weeks and divided into two categories, those that did not develop learned helplessness or recovered during the 4 weeks of testing (NLH) and those that developed learned helplessness and remained learned helpless for 4 weeks (LH; failed to escape >15 out of the 30 trials every week). Just after the last foot shocks of the 4th week of testing, mice were sacrificed, intestines were recovered, and RNA extracted. Expression of SAA1 (F), SAA2 (G), SAA3 (H), IL-22 (I) and GAPDH were measured by qRT-PCR. Each symbol represents an individual mouse. Mean $\pm$ SEM, n=4–12 mice/group, one-way ANOVA, F(2,24)= 4.256 (F), F(2,24)=4.66 (G), * p <0.05 Bonferroni's post-hoc test.

Figure 5: Oleic acid exhibits antidepressant properties.

TAC mice were injected with 5 nmoles/mouse of oleic acid or vehicle, i.p. daily for 2 weeks, and on the 3 last days of injection some mice received 5 nmoles/mouse of AI-2 or vehicle, i.p. daily for the 3 last days. On the last day of treatment mice were subjected to the learned helplessness paradigm. Levels of AI-2 (A), sc-AHL (B), and lc-AHL (C) were measured in the stools immediately after the last foot shock. Each symbol represents an individual mouse. Data are means $\pm$ SEM. n=8–10/group, Mann-Whitney, U=13, *p<0.05. (D) Escape failures were recorded and means $\pm$ SEM of the escape failures are displayed, and the percent of mice exhibiting learned helplessness (failing to escape >15 out of the 30 trials) is shown above each group. Each symbol represents an individual mouse. n=5–14/group, One-way ANOVA, F(2, 31)=6.054, *p<0.05 Bonferroni post-hoc test. Intestinal expression of SAA1

(E), SAA2 (F), SAA3 (G), IL-22 (H) and GAPDH were measured by qRT-PCR. Each symbol represents an individual mouse. Mean $\pm$ SEM, n=4–14, one-way ANOVA, F(2,26)=3.809 *p<0.05 (E), F(2,26)=6.958 *p<0.05 (F). Th17 (I), Th1 (J) and Tregs (K) cells were analysed in the hippocampus. Each symbol represents an individual mouse. Data are means $\pm$ SEM. n=9–10/group, Mann-Whitney, U=23, *p<0.05. L, Schematic conclusion.

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Figure 6: Patients with current MDD exhibit dysregulation of the SFB/AI-2/SAA/IL-17A circuit. Levels of IL-17A (A), SFB (B), SAA (including all human isoforms), C), AI-2 (D), sc-AHL (E) and lc-AHL (F) were analysed in fecal MDD and healthy control (HC) samples using multiplex approach (A-C), qRT-PCR (B) or reporter assay (D-F). N=8-9, Mann-Whitney, U=8, *p=0.0065 (A), N=7-8, Unpaired *t* test, *t*=2.249, *p=0.0425 (B), N=7-8, Unpaired *t* test, *t*=2.461, *p=0.0286 (C), N=10 (D-F).

Table 1:
Pearson regression analyses for AI-2, SFB, SAA, IL-17A and QIDS in patients with current MDD and healthy subjects.

AI-2, autoinducer-2, SFB, Segmented filamentous bacteria, SAA, Serum Amyloid protein, IL-17A, Interleukin-17A, QIDS, Quick Inventory for Depressive Symptomatology

	<i>p</i> -value	Adjusted r^2	<i>r</i>	N
AI-2/SFB	0.019	0.196	0.494	18
SFB/SAA	0.004	0.336	0.614	18
SAA/IL-17A	0.011	0.288	0.582	15
IL-17A/QIDS	0.013	0.245	0.540	17