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Immunization with a heat-killed preparation of the environmental bacterium *Mycobacterium vaccae* promotes stress resilience in mice

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The prevalence of inflammatory diseases is increasing in modern urban societies. Inflammation increases risk of stress-related pathology; consequently, immunoregulatory or antiinflammatory approaches may protect against negative stress-related outcomes. We show that stress disrupts the homeostatic relationship between the microbiota and the host, resulting in exaggerated inflammation. Repeated immunization with a heat-killed preparation of *Mycobacterium vaccae*, an immunoregulatory environmental microorganism, reduced subordinate, flight, and avoiding behavioral responses to a dominant aggressor in a murine model of chronic psychosocial stress when tested 1–2 wk following the final immunization. Furthermore, immunization with *M. vaccae* prevented stress-induced spontaneous colitis and, in stressed mice, induced anxiolytic or fear-reducing effects as measured on the elevated plus-maze, despite stress-induced gut microbiota changes characteristic of gut infection and colitis. Immunization with *M. vaccae* also prevented stress-induced aggravation of colitis in a model of inflammatory bowel disease. Depletion of regulatory T cells negated protective effects of immunization with *M. vaccae* on stress-induced colitis and anxiety-like or fear behaviors. These data provide a framework for developing microbiome- and immunoregulation-based strategies for prevention of stress-related pathologies.

anxiety | chronic psychosocial stress | fear | microbiota | posttraumatic stress disorder

Immunoregulation, indicated by a balanced expansion of effector T-cell populations and regulatory T cells (Treg), is known to be driven by microbial signals, mainly by organisms with which mammals coevolved, including: (*i*) the commensal microbiota, which have been altered by the Western lifestyle, including a diet that is commonly low in microbiota-accessible carbohydrates (1, 2); (*ii*), pathogens associated with the "old infections" that were present throughout life in evolving human hunter-gatherer populations (3); and (*iii*) organisms from the natural environment with which humans were inevitably in daily contact (and so had to be tolerated by the immune system) (4). Immunoregulation is thought to be compromised in modern high-income settings due to reduced contact with these three categories of organisms (4–6). A failure of immunoregulation, attributable to reduced exposure to the microbial environment within which the mammalian immune system evolved, is thought to be one factor contributing to recent increases in stress-related and chronic inflammatory disorders in high-income countries (1, 3, 4). Results from both preclinical and clinical studies are consistent with the idea that inadequate immunoregulation also increases risk for development of stress-related psychiatric disorders (4, 7, 8).

Consistent with the hypothesis that subjects with stress-related psychiatric disorders, such as posttraumatic stress disorder (PTSD), suffer from a failure of immunoregulation, PTSD is associated with decreases in Treg (9), an increased proinflammatory milieu (10), autoimmunity (11), and exaggerated symptoms of inflammatory bowel disease (IBD) (11, 12). Prospective studies have demonstrated that elevated plasma concentrations of C-reactive protein predict subsequent PTSD symptoms (7). Furthermore, prospective studies

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Significance

The hygiene, or "old friends," hypothesis proposes that lack of exposure to immunoregulatory microorganisms in modern urban societies is resulting in an epidemic of inflammatory disease, as well as psychiatric disorders in which chronic, low-level inflammation is a risk factor. An important determinant of immunoregulation is the microbial community occupying the host organism, collectively referred to as the microbiota. Here we show that stress disrupts the homeostatic relationship between the microbiota and the host, resulting in exaggerated inflammation. Treatment of mice with a heat-killed preparation of an immunoregulatory environmental microorganism, *Mycobacterium vaccae*, prevents stress-induced pathology. These data support a strategy of "reintroducing" humans to their old friends to promote optimal health and wellness.

of gene networks identify enrichment of innate immune responses and IFN signaling (types I and II) as putative causal signatures for PTSD development (13).

Trauma and stressor exposure can alter the composition of the gut microbiome (14) and, consequently, the homeostatic balance between the gut microbiota and mucosal immune system, with important consequences for enteric infections, mucosal inflammation, bacterial translocation (15), as well as emotional behavior, including anxiety-like behavior (16). Glucocorticoid hormones, important mediators of physiologic responses to stress, increase the abundance of pathobionts, decrease IgA (which normally inhibits bacterial adherence to intestinal epithelial cells), increase bacterial adherence over twofold, and increase bacterial translocation to mesenteric lymph nodes (17, 18). Furthermore, stress-induced decreases in an individual's microbial diversity are thought to increase vulnerability to infectious pathology (15). Meanwhile, orally administered probiotics with immunoregulatory and antiinflammatory properties have been shown to induce anxiolytic and antidepressant-like effects in animal models (6, 16). It remains unclear whether these beneficial effects of probiotics are due to their ability to prevent stress-induced decreases in microbial diversity, their immunoregulatory effects, or both.

Previous studies have demonstrated that probiotics can have antiinflammatory effects in rodent models of chronic inflammation, including colitis, following either mucosal or subcutaneous administration (19, 20), and in some cases these effects are observed using heat-killed preparations (20). Subcutaneous injections of heat-killed preparations of immunoregulatory bacteria may have some advantages, including long-term duration of antiinflammatory and immunoregulatory effects, lasting up to 12 wk following administration (21).

If inadequate immunoregulation and subsequent chronic low-grade inflammation are risk factors for development of stress-related psychiatric disorders, pretreatment with an immunoregulatory agent would be expected to be protective. However, the potential for immunoregulatory approaches to prevent stress-related psychiatric disorders has not been tested. Therefore, in the current study, we evaluated the potential for immunization with a heat-killed preparation of *Mycobacterium vaccae* to prevent chronic psychosocial stress-induced pathophysiology, including spontaneous colitis, exaggeration of chemically induced colitis, and exaggerated anxiety- and fear-like behaviors. *M. vaccae* is an abundant soil saprophyte, a microorganism that lives on dead or decaying organic matter, with immunoregulatory properties (22). A heat-killed preparation of the organism modulates dendritic cell function (23) and induces Treg and secretion of antiinflammatory cytokines, including IL-10 and transforming growth factor β (22).

Results

***M. vaccae* Increases Proactive Coping.** Reactive, as opposed to proactive, coping behavior may increase the risk of developing stress-related disorders in humans (24) and anxiety- and depressive-like responses in rodents (25). Here we quantified reactive versus proactive coping responses during exposure to the chronic subordinate colony housing (CSC) procedure (26) (Exp. 1) (for details, see *SI Materials and Methods*; Fig. S1 *A* and *B*). Briefly, we immunized male C57BL/6NCrl mice with either vehicle or a heat-killed preparation of *M. vaccae* [National Collection of Type Cultures (NCTC) 11659; 0.1 mg, subcutaneously] (Fig. S2*A*) on days –21, –14, and –7. On day 1, mice were assigned to the single-housed control (SHC) group or CSC group, with four CSC mice being housed together with a dominant male for 19 consecutive days. We assessed stress coping behaviors of *M. vaccae*- or vehicle-immunized mice during 2 h of CSC exposure on days 1, 8, and 15, effects of preimmunization with *M. vaccae* on CSC-induced changes in the gut microbiome on days –21, –14, –7, 1, 8, and 15, anxiety-like behavior on the elevated plus-maze (EPM) on day 19, and pathophysiology on day 20.

M. vaccae immunization did not affect body weight gain before CSC exposure (vehicle, 6.4 ± 0.3 g; *M. vaccae*, 6.9 ± 0.3 g; Student's *t* test, $P > 0.05$) and did not affect CSC-induced reduction in body weight gain (Fig. S2*B*). However, immunization with *M. vaccae* decreased the number of submissive upright posture displays (Fig. 1*A*) [linear mixed model (LMM) for AM behavior, *M. vaccae* × time, $F_{(2,\ 93.0)} = 9.6$, $P < 0.001$]. These effects were particularly evident during the first hour of CSC exposure on day 1, when *M. vaccae*-immunized mice showed a 63% reduction in the amount of submissive upright posture relative to vehicle-injected mice (Fig. 1*A*). Whereas 95.7% of vehicle-injected mice were defeated, as measured by displaying at least one submissive upright posture, only 65.2% of *M. vaccae*-immunized mice were defeated during the first hour on day 1 (Fisher's exact test, $P < 0.05$). *M. vaccae*-immunized mice also showed reduced numbers of flight and avoiding behaviors (Fig. S2*C*) [LMM for AM behavior, *M. vaccae*, $F_{(1,\ 131.5)} = 10.8$, $P < 0.01$]. There were no differences in the number of times experimental CSC mice attacked or chased the resident male (Fig. S2 *D* and *E*). *M. vaccae*-treated mice had a higher dominance index [the sum of proactive behaviors (attacks, chasing) minus the sum of reactive behaviors (submissive upright postures, flight, avoiding)] (Fig. 1*B*) [LMM for AM behavior, *M. vaccae* × time, $F_{(2,\ 90.2)} = 4.5$, $P < 0.05$]. Overall, during the 19-d CSC procedure, 69.6% of *M. vaccae*-immunized mice displayed at least one proactive behavior, whereas only 21.7% of vehicle-treated mice did so (Fig. 1*C*) (Fisher's exact test, $P < 0.01$).

A nearly identical pattern of behavior was observed during CSC exposure when the interval between the final immunization and CSC exposure was extended to 2 wk (Figs. S1*C* and S2 *F*–*K*). Immunization with *M. vaccae* decreased the number of submissive upright posture displays (Fig. S2*F*) [LMM, *M. vaccae*, $F_{(1,\ 37.6)} = 14.9$, $P < 0.001$]. *M. vaccae*-immunized mice also showed reduced numbers of flight and avoiding behaviors (Fig. S2*G*) [LMM for AM behavior, *M. vaccae* × time, $F_{(2,\ 28.8)} = 10.5$, $P < 0.001$]. There were no differences in the number of times experimental CSC mice attacked or chased the resident male (Fig. S2 *H* and *I*). *M. vaccae*-treated mice had a higher dominance index (Fig. S2*J*) [LMM for AM behavior, *M. vaccae* × time, $F_{(2,\ 30.2)} = 14.8$, $P < 0.0001$]. Overall, during the 19-d CSC procedure, 62.5% of *M. vaccae*-immunized mice displayed at least one proactive behavior, whereas only 25.0% of vehicle-treated mice did so (Fig. S2*K*) (Fisher's exact test, $P = 0.14$).

Together, these data demonstrate that immunization with *M. vaccae* induced a long-lasting shift toward a more proactive coping response (27), characterized by decreased submissive, flight, and avoiding behaviors, during chronic psychosocial stress that, based on previous studies in rodents and humans, may decrease vulnerability to the development of more persistent anxiety- and depressive-like symptoms (24, 25).

When tested on day 19, following the 19-d CSC procedure, CSC exposure had anxiolytic or fear-reducing effects in *M. vaccae*-immunized mice but not vehicle-immunized mice, as measured by

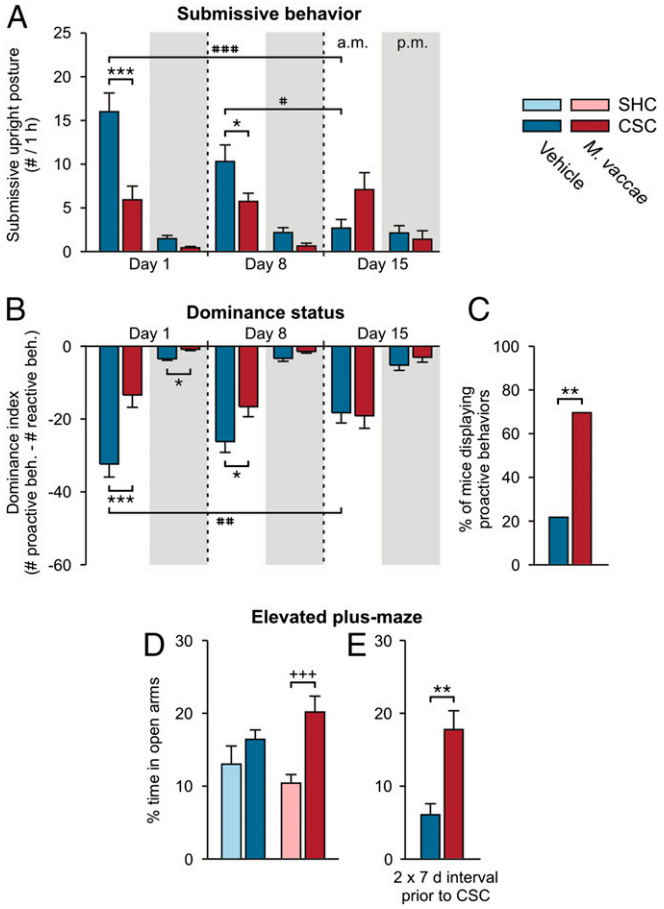

Fig. 1. Immunization with heat-killed *M. vaccae* induces proactive stress coping during chronic subordinate colony housing exposure and anxiolytic or fear-reducing behavioral responses on day 19. (*A*) Number of submissive upright posture displays (10–11:00 AM, white background; 5–6:00 PM, gray background) on days 1, 8, and 15 of CSC. (*B*) Dominance index. (*C*) Percent of vehicle- and *M. vaccae*-immunized mice displaying proactive behaviors during the 19-d CSC procedure. (*D* and *E*) Anxiety-like or fear-reducing behavior as measured on the elevated plus-maze on day 19 in (*D*) Exp. 1 or (*E*) Exp. 2. Bars represent means; error bars represent +SEM (*A* and *C*–*E*) or –SEM (*B*). Significance was assessed by linear mixed model analysis, conducted separately for AM and PM time points (*A* and *B*), Fisher's exact test (*C*), two-factor ANOVA (*D*), and Student's *t* test (*E*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, (*A* and *B*) between-subjects effects of *M. vaccae* versus vehicle, Fisher's least significant difference (LSD) tests; (*C*) Fisher's exact test; (*E*) Student's *t* test. $^{+++}P < 0.01$, (*D*) between-subjects effects of SHC versus CSC, Fisher's LSD test; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, (*A* and *B*) within-subjects effects of *time*, paired *t* tests using Bonferroni correction. The number of independent data points (*N*) in each of the graphs and sample size (*n*) for each group are as follows: (*A* and *B*) *N* = 46; vehicle, 22–23; *M. vaccae*, 22–23. (*C*) *N* = 46; vehicle, 23; *M. vaccae*, 23. (*D*) *N* = 47; vehicle/SHC, 7; vehicle/CSC, 15; *M. vaccae*/SHC, 9; *M. vaccae*/CSC, 16. (*E*) *N* = 16; vehicle/CSC, 8; *M. vaccae*/CSC, 8.

time spent on the open arms of the EPM (Fig. 1*D* and Table S1) [two-factor ANOVA, *M. vaccae* × CSC, $F_{(1,\ 43)} = 2.3$, $P = 0.13$; CSC, $F_{(1,\ 43)} = 10.1$, $P < 0.01$]. *M. vaccae*-immunized, CSC-exposed mice spent more time exploring the aversive open arms of the EPM relative to *M. vaccae*-immunized, SHC mice. In Exp. 2, when a 2-wk interval was used between the final immunization with *M. vaccae* and the onset of the CSC procedure, *M. vaccae* immunization induced a strong anxiolytic response when CSC-exposed mice were tested on the EPM on day 20 (Fig. 1*E*, Fig. S1*C*, and Table S1) [Student's *t* test, $t_{(1,\ 14)} = 3.9$, $P < 0.01$]. In contrast to our previous data (28), CSC exposure did not increase anxiety-like behavior in vehicle-treated mice (Fig. 1*D*), probably representing a floor effect (vehicle-treated mice spent very little time exploring the open arms); vehicle-treated mice in the current study received multiple subcutaneous injections and were older at the time of testing, relative to previous studies. These differences may explain the high baseline anxiety in vehicle-immunized mice.

In Exp. 3, CSC exposure had an anxiogenic effect in the social preference/avoidance test, decreasing time spent in the contact zones of the novel object and novel conspecific (Figs. S1*D* and S2*L*) [LMM, CSC, $F_{(1,\ 43.3)} = 7.3$, $P < 0.05$]. There was an overall preference for social contact, relative to the novel object (Fig. S2*L*) [LMM, social, $F_{(1,\ 45.8)} = 11.1$, $P < 0.01$]. There were no effects of *M. vaccae*, or *M. vaccae* × CSC interactions, on conflict anxiety in the social preference/avoidance test, and there were no effects of either *M. vaccae* immunization or CSC exposure on locomotor activity (Fig. S2*M*). There were no effects of either *M. vaccae* immunization or CSC exposure on conflict anxiety or locomotor activity in the light/dark box test (Figs. S1*D* and S2 *N* and *O*).

Consistent with previous studies, CSC exposure increased adrenal weight (Figs. S1*B* and S2*P*) and in vitro adrenal insensitivity to adrenocorticotropic hormone (ACTH) (Figs. S1*B* and S2*Q*). *M. vaccae* immunization did not prevent these effects. These data suggest that CSC exposure was physically and/or psychologically stressful for both vehicle- and *M. vaccae*-immunized groups.

Persistent Effects of *M. vaccae* Immunization on Brain Serotonergic Systems. Because chronic exercise alters brain serotonergic gene expression (29–31) and because this may be relevant to the stress resistance effects of chronic exercise, we examined the effects of CSC exposure and *M. vaccae* immunization on serotonergic gene expression in the brainstem raphe nuclei. Specifically, we analyzed expression of *tph2*, encoding tryptophan hydroxylase 2, the rate-limiting enzyme in the biosynthesis of serotonin, and *slc6a4*, encoding solute carrier family 6 (neurotransmitter transporter), member 4, the high-affinity, low-capacity, sodium-dependent serotonin transporter. Immunization with *M. vaccae* increased *tph2* mRNA expression selectively in the rostral region of the dorsal raphe nucleus, dorsal part (rDRD) (Fig. S3 *A*–*D* and Table S1) [LMM, *M. vaccae* × region, $F_{(8,\ 715.6)} = 7.4$, $P < 0.001$].

Immunization with *M. vaccae* also prevented a stress-induced decrease in *slc6a4* mRNA expression, also in the rDRD (Fig. S3*E*) [two-factor ANOVA, CSC, $F_{(1,\ 27)} = 6.5$, $P < 0.05$], further supporting long-term effects of *M. vaccae* immunization on this subset of serotonergic neurons. Broader implications of these findings include the capacity for bioimmunomodulatory approaches to alter gene expression patterns in highly specific neural systems in the brain across a long timescale, at least 4 wk, effects that may influence stress coping strategies and stress resilience.

Influence of *M. vaccae* Immunization on Microglia. Given the potential role of inflammatory mediators in determining behavioral coping responses to psychosocial stressors (32) and recent findings that the gut microbiota and peripheral immune activity continuously control maturation and function of microglia in the central nervous system (CNS) (33, 34), we investigated the effects of *M. vaccae* immunization on microglial density. To determine whether *M. vaccae* immunization altered the number or morphological properties of microglia within the brain, we performed immunohistochemical staining of ionized calcium-binding adapter molecule 1 (Iba1), a 17-kDa actin-binding protein specifically and constitutively expressed in microglia (35, 36) that is an immunohistochemical marker for both ramified and activated microglia (35, 37). Iba1 immunostaining was evaluated in brain regions implicated in control of anxiety and fear states (Fig. S3*F*). Because we were interested in effects of *M. vaccae* on activated microglia, we conducted analyses using CSC-exposed mice only. The density and morphology of microglia were analyzed using image analysis. We found that, among CSC-exposed mice, immunization with *M. vaccae* selectively increased microglial density in the prelimbic

part of the medial prefrontal cortex (PrL) (Fig. S3 *G* and *I*) [Student's *t* test, $t_{(1, 24)} = 2.4$, $P < 0.05$], which plays an important role in fear expression (38) and provides the main cortical input to the dorsal raphe nucleus controlling stress-induced anxiety states (39). There were no effects in the infralimbic part (IL) of the medial prefrontal cortex or other regions studied. Detailed cumulative densitometric threshold analysis revealed that there was 8% more Iba1 immunostaining in the PrL in *M. vaccae*-immunized CSC-exposed mice compared with vehicle-immunized CSC-exposed mice (Fig. S3 *H* and *I*) [Student's *t* test, 56.1 ± 2.3 versus 48.1 ± 2.4, respectively, $t_{(1, 24)} = 3.1$, $P < 0.01$]. These data confirm the long-term effects of *M. vaccae* immunization on the microglial phenotype in the PrL, a forebrain structure critical for control of fear expression, in CSC-exposed mice.

Stress Promotes Colitogenic Dysbiosis. Given that stress alters the composition of the gut microbiome (14) and, consequently, the homeostatic balance between the gut microbiota and mucosal immune system, with important consequences for mucosal inflammation (15) as well as emotional behavior, including anxiety-like behavior (16), we conducted next-generation sequencing to characterize the effects of stress on the composition of the gut microbiome. Furthermore, we investigated whether or not prior immunization with *M. vaccae* had any impact on stress-induced changes in the gut microbiota. In microbial ecology, there are two principal measures of species diversity, with α-diversity assessing diversity within a sample and β-diversity assessing diversity between samples. There were strong overall declines in α-diversity over time, particularly evident at the onset of the CSC procedure (Fig. S4 *A–C*) [LMM, day, phylogenetic diversity, $F_{(5, 28.1)} = 11.0$, $P < 0.0001$; observed species, $F_{(5, 28.5)} = 10.9$, $P < 0.0001$; Shannon index, $F_{(5, 35.9)} = 13.9$, $P < 0.0001$], suggesting that the CSC procedure was stressful for all mice, which were housed in the same room. As observed with other stressors (15), CSC exposure increased β-diversity, relative to SHC conditions (Fig. S4 *D* and *E*). Based on analysis of all samples across all time points, α-diversity was higher and β-diversity was lower in *M. vaccae*-immunized mice compared with vehicle-immunized mice (Fig. S4 *A–E*) [LMM, α-diversity, *M. vaccae*, phylogenetic diversity, $F_{(1, 26.9)} = 5.9$, $P < 0.05$; observed species, $F_{(1, 31.1)} = 5.4$, $P < 0.05$; Shannon index, $F_{(1, 37.0)} = 11.8$, $P < 0.01$; *M. vaccae* × CSC × day, phylogenetic diversity, $F_{(16, 36.1)} = 2.1$, $P < 0.05$; observed species, $F_{(16, 36.1)} = 2.1$, $P < 0.05$; Shannon index, $F_{(16, 47.5)} = 1.3$, $P = 0.27$], indicating that *M. vaccae* immunization had a stabilizing effect on the gut microbiota throughout the study, consistent with recent studies demonstrating that host adaptive immunity modulates the gut microbiota (40). In line with these findings, multiple linear regression showed that 11% of the variation in the gut microbiota was explained by the histological damage score in the colon, reflecting intestinal immune activation.

Detailed analysis of the microbial composition, conducted using analysis of composition of microbiomes (ANCOM) (41), revealed main effects of CSC to increase the abundance of Proteobacteria (percent relative abundances are plotted in Fig. S4*F*), including *Helicobacter*, and an unidentified genus of Helicobacteraceae, as well as *Paraprevotella* (Bacteroidetes) on day 8 or 15 [ANCOM, false discovery rate (FDR)-adjusted $P < 0.05$] (Figs. S4 *G–J* and S5 *A–C* and Table S2). Changes in microbial community structure over time were evaluated using ANCOM over all six time points, followed, when appropriate, by Wilcoxon signed-rank tests comparing days 1 versus 8 and days 1 versus 15, using Bonferroni correction (Fig. S4*K*). Consistent with the analysis of the main effects of CSC exposure above, time-dependent increases in *Helicobacter*, two unidentified genera of Helicobacteraceae, and *Paraprevotella* were seen on days 8 and 15 in both vehicle-immunized and *M. vaccae*-immunized CSC mice but not SHC mice (Fig. S5 *M–O* and *S* and Table S3). In addition, decreases in *Mucispirillum* were observed on days 8 and 15 in both vehicle-immunized and *M. vaccae*-immunized CSC mice but not SHC mice (Fig. S5*Q* and Table S3). Furthermore, we observed a main effect of *M. vaccae* to stabilize the abundance of several genera on day 8 or 15, including an unidentified genus of Desulfovibrionaceae (Proteobacteria) (Fig. S5*X*). Our data are consistent with a CSC-induced gut dysbiosis and a shift toward a gut microbiota with increased potential for inflammation (Fig. S4*H*). Psychological stress increases *Helicobacter* abundance through glucocorticoid actions on glucocorticoid receptors (18), and previous studies have found that *Helicobacter* abundance predicts intestinal inflammation scores specifically in mice with impaired immunoregulation (IL-$10^{-/-}$ mice; $r = 0.58$) (42). Consistent with these previous findings, relative abundances of both Proteobacteria and *Helicobacter* predicted histological damage to the colon in our study (Fig. S4 *L* and *M*). Meanwhile, expansion of *Paraprevotella*, as observed in our study (Figs. S4 *H* and *I* and S5*S*), has been associated with multiple murine models of experimental autoimmunity (43), consistent with a stress-induced autoimmune-like response to dietary, microbiota, or self-antigens in the absence of adequate immunoregulation. Finally, decreases in the abundance of *Mucispirillum* over time, as observed in our study in both vehicle/CSC and *M. vaccae*/CSC mice (Figs. S4 *H* and *J* and S5*Q* and Table S3), have been identified as a biological signature of gut infection (44). A decline of *Mucispirillum* is associated with early disruption of the colonic surface mucus layer and a prolonged delay to recovery after the period of pathogen clearance (44).

***M. vaccae* Prevents Stress-Induced Colitis.** Evidence suggests that anxiety and depression are more common in IBD patients and that the symptoms of these conditions are more severe during periods of active disease (45). Oral or intraperitoneal administration of immunomodulatory bacterial products have been shown to both prevent and treat experimental colitis in animal models (46, 47), suggesting that these substances can act through gut-dependent and gut-independent mechanisms to attenuate chemically induced colitis. Chronic subordinate colony housing exposure reproducibly induces spontaneous colitis and aggravates chemically induced colitis (26). Importantly, although immunization with *M. vaccae* did not prevent the CSC-induced increase in colitogenic *Helicobacter* spp. (Figs. S4*H* and S5 *B* and *M–O* and Table S3), it did prevent CSC-induced spontaneous colitis [Fig. 2 *A* and *B*; Exp. 1, two-factor ANOVA, *M. vaccae* × CSC, $F_{(1, 29)} = 6.7$, $P < 0.05$; Fig. 2*B*, Exp. 2, Student's *t* test, $t_{(1, 14)} = 5.4$, $P < 0.05$], and decreased plasma concentrations of kynurenine, a biomarker of inflammation (48) (Fig. S2*R*) [LMM, *M. vaccae*, $F_{(1, 24)} = 5.6$, $P < 0.05$], suggesting increased immunoregulation (49). In contrast, CSC exposure decreased tryptophan concentrations (Fig. S2*S*) [two-factor ANOVA, CSC, $F_{(1, 27)} = 4.705$, $P < 0.05$], whereas *M. vaccae* immunization had no effect.

Similarly, *M. vaccae* pretreatment prevented the CSC-induced aggravation of dextran sulfate sodium (DSS; 1% for 7 d)-induced colitis (Exp. 3) (Fig. 2 *C–I* and Fig. S1*D*) [two-factor ANOVA, CSC, $F_{(1, 26)} = 4.6$, $P < 0.05$]. Of note, in the model of DSS-induced colitis, *M. vaccae* immunization attenuated the CSC-induced increase in the number of viable mesenteric lymph node cells (Fig. 2*E*) [two-factor ANOVA, *M. vaccae*, $F_{(1, 24)} = 4.8$, $P < 0.05$; CSC, $F_{(1, 24)} = 76.4$, $P < 0.0001$] and attenuated the CSC-induced IFN-γ (Fig. 2*F*) [two-factor ANOVA, CSC, $F_{(1, 24)} = 8.5$, $P < 0.01$] and IL-6 secretion [Fig. 2*G*; two-factor ANOVA, *M. vaccae* × CSC, $F_{(1, 24)} = 14.9$, $P < 0.01$]. Chronic subordinate colony housing exposure increased IL-10 secretion from anti-CD3–stimulated mesenteric lymph node cells assessed in vitro (Fig. 2 *H* and *I*) [two-factor ANOVA, CSC, $F_{(1, 24)} = 12.7$, $P < 0.01$], an effect that was only evident in *M. vaccae*-immunized mice, indicating an antiinflammatory bias in *M. vaccae*-immunized, CSC-exposed mice. Overall, these data suggest that, in the absence of adequate immunoregulation, CSC exposure led to activation of the host immune response toward

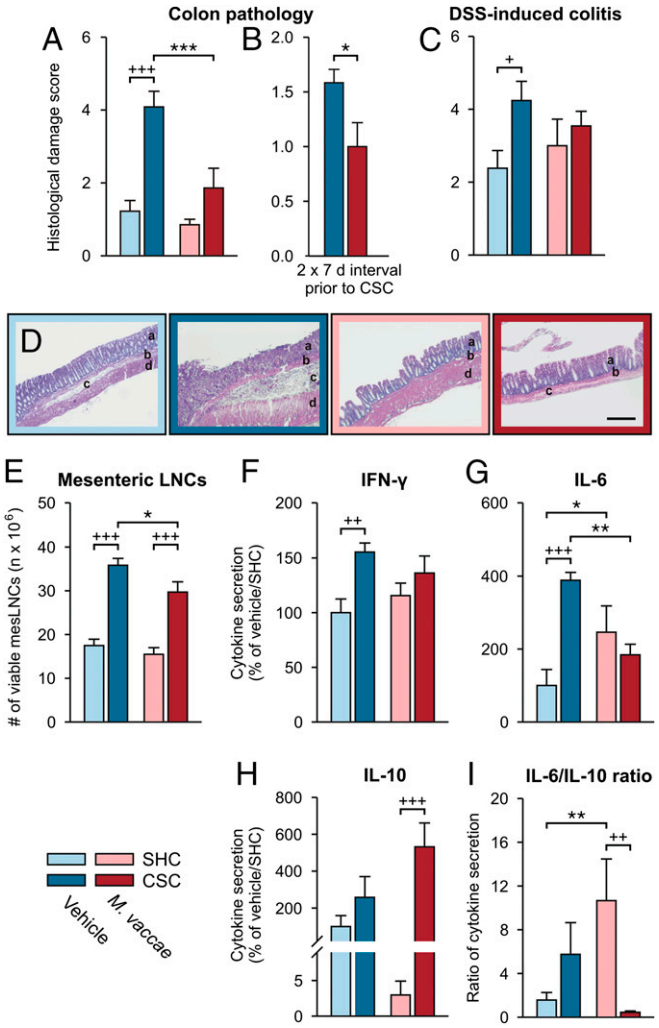

Fig. 2. *M. vaccae* prevents chronic CSC-induced spontaneous colitis and CSC-induced aggravation of chemically induced colitis. (*A* and *B*) Colonic histological damage scores reflecting CSC-induced spontaneous colitis on day 20 of (*A*) Exp.1 and (*B*) Exp. 2. (*C–I*) CSC-induced aggravation of chemically induced colitis on day 29 of Exp. 3. (*C*) Colonic histological damage scores following SHC or CSC conditions, followed by administration of DSS (1%; days 22–29) in drinking water. (*D*) Photomicrographs from hematoxylin and eosin-stained colon sections. (Scale bar, 200 μm.) a, lamina mucosa; b, lamina muscularis mucosa; c, lamina submucosa; d, lamina muscularis externa. (*E*) Number of viable mesenteric lymph node cells (mesLNCs). (*F–H*) IFN-γ (*F*), IL-6 (*G*), and IL-10 (*H*) secretion from mesLNCs stimulated with anti-CD3 antibody in vitro. (*I*) IL-6/IL-10 ratio. Bars represent means; error bars represent +SEM. Significance was assessed using (*A*, *C*, and *E–I*) two-factor ANOVA and (*B*) Student's *t* test. Post hoc comparisons were made using Fisher's LSD tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, between-subjects effects of vehicle versus *M. vaccae*, within the same CSC/SHC condition; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, between-subjects effects of SHC versus CSC, within the same drug condition. The number of independent data points (*N*) in each of the graphs (*A–C* and *E–I*) and sample size (*n*) for each group are as follows: (*A*) *N* = 33; vehicle/SHC, 9; vehicle/CSC, 8; *M. vaccae*/SHC, 9; *M. vaccae*/CSC, 7. (*B*) *N* = 16; vehicle/CSC, 8; *M. vaccae*/CSC, 8. (*C*) *N* = 30; vehicle/SHC, 7; vehicle/CSC, 7; *M. vaccae*/SHC, 8; *M. vaccae*/CSC, 8. (*E*, *F*, and *H*) *N* = 28; vehicle/SHC, 6; vehicle/CSC, 7; *M. vaccae*/SHC, 7; *M. vaccae*/CSC, 8. (*G* and *I*) *N* = 27; vehicle/SHC, 6; vehicle/CSC, 6; *M. vaccae*/SHC, 7; *M. vaccae*/CSC, 8.

Helicobacter spp., other elements of the CSC-induced colitogenic microbiota, or dietary or self-antigens, resulting in colitis. Furthermore, these data suggest that immunization with *M. vaccae* restored immunoregulation and prevented colitogenic effects of CSC, despite stress-induced development of a gut microbiota with colitogenic potential. Based on these findings, immunization with *M. vaccae*, or similar bioimmunomodulatory approaches, may be useful for prevention of chronic stress/repeated trauma-induced inflammation and subsequent development of somatic and mental disorders.

Treg Dependence of *M. vaccae* Effects. Psychosocial stress decreases Treg in mice and humans (50, 51), and Treg depletion increases anxiety- and depressive-like behaviors in mice (52). Likewise, PTSD subjects (9) and subjects with major depressive disorder (53) have reduced Treg, which is reversed 1 y following effective narrative exposure therapy (54) or treatment with antidepressants, respectively (53). Given these findings and studies showing that CSC reduces Treg in peripheral lymph nodes (26) and *M. vaccae* immunization induces Treg (22), we investigated a potential role for Treg in the stress-protective effects of *M. vaccae* (Exp. 4) (Fig. S1*E*). Here, we were primarily interested in the effects of depletion of Treg in *M. vaccae*-immunized mice. Therefore, all mice were immunized with *M. vaccae* and on day –4 treated intraperitoneally with either anti-CD25 antibody [PC-61.5.3; administration of this anti-CD25 antibody is an effective means of depleting Treg in mice in vivo (55, 56)] or control antibody [rat IgG1 isotype control; anti-horseradish peroxidase (HRPN)] (Fig. S1*E*; for confirmation of the efficacy of Treg depletion, see Fig. S6 *A–C*). As before, we assessed dominant–subordinate interactions during CSC exposure. On day 19 of CSC, mice were tested on the EPM, and then, on day 20, mice were euthanized for collection of adrenals, colon, and mesenteric lymph nodes.

Among *M. vaccae*-immunized mice, treatment with anti-CD25 antibody had no effect on stress coping behaviors during CSC (Fig. S6 *D–I*). Treatment of *M. vaccae*-immunized mice with anti-CD25 antibody had no effect on *tph2* or *slc6a4* mRNA expression in the rDRD (Table S1), suggesting that both the effects of *M. vaccae* on behavioral coping strategies during CSC exposure and brain *tph2* and *slc6a4* mRNA expression are independent of Treg. Interestingly, treatment with the anti-CD25 antibody, which would be expected to increase proinflammatory signaling in the periphery, increased *tph2* mRNA expression in the interfascicular part of the dorsal raphe nucleus (DRI) (Fig. S6*L*) [two-factor ANOVA, anti-CD25, $F_{(1,\ 22)} = 4.7$, $P < 0.05$], a subpopulation of serotonergic neurons that we have shown previously is activated by acute proinflammatory stimuli (57, 58).

Stress-induced adrenal hypertrophy was evident in *M. vaccae*-immunized CSC mice treated with either control or anti-CD25 antibody (Fig. S6*J*) [two-factor ANOVA, CSC, $F_{(1,\ 27)} = 29.7$, $P < 0.0001$], as was the stress-induced adrenal ACTH insensitivity (Fig. S6*K*) [three-factor ANOVA, CSC, $F_{(1,\ 52)} = 33.2$, $P < 0.0001$], consistent with results from Exp. 1, indicating that CSC exposure was aversive for all animals and that *M. vaccae* does not affect CSC-induced changes in hypothalamic–pituitary–adrenal (HPA) axis function.

Anti-CD25 antibody treatment of *M. vaccae*-immunized, CSC-exposed mice prevented the permissive effect of *M. vaccae* immunization on CSC-induced reductions in anxiety, as assessed by the percentage of time spent on the open arms of the EPM (Fig. 3*A*; Table S1) [two-factor ANOVA, anti-CD25 × CSC, $F_{(1,\ 21)} = 7.4$, $P < 0.05$], suggesting that the anxiolytic or fear-reducing effects of *M. vaccae* are dependent on Treg. As expected, there was also no CSC-induced colitis in *M. vaccae*-immunized mice pretreated with control antibody, whereas *M. vaccae*-immunized, CSC-exposed mice pretreated with anti-CD25 antibody responded to CSC exposure with development of spontaneous colitis (Fig. 3 *B–G*) [based on anti-CD25 × CSC interactions using two-factor ANOVAs for histological damage score: $F_{(1,\ 23)} = 14.6$, $P < 0.01$; number of viable mesenteric lymph node cells: $F_{(1,\ 27)} = 6.2$, $P < 0.05$; and anti-CD3–stimulated cytokine secretion from mesenteric lymph node cells in vitro, IFN-γ: $F_{(1,\ 28)} = 20.8$, $P < 0.0001$; IL-6: $F_{(1,\ 27)} = 4.9$, $P < 0.05$; and IL-10: $F_{(1,\ 26)} = 8.6$,

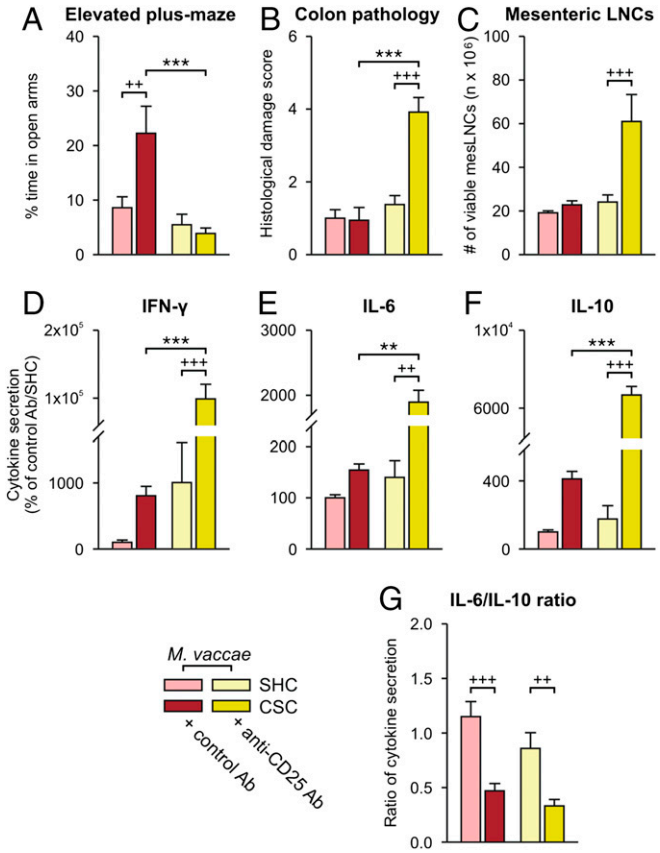

Fig. 3. Depletion of regulatory T cells prevents stress-protective effects of *M. vaccae*. (*A*) Anxiety- or fear-like behavior on the elevated plus-maze (day 19; Exp. 4). (*B–G*) CSC-induced spontaneous colitis measured on day 20. (*B*) Colonic histological damage scores. (*C*) Number of viable mesLNCs. (*D–F*) Secretion of (*D*) IFN-γ, (*E*) IL-6, and (*F*) IL-10 from mesLNCs stimulated with anti-CD3 antibody in vitro (control antibody/SHC group: 100%). (*G*) IL-6/IL-10 ratio. Bars represent means; error bars represent +SEM. Significance was assessed using two-factor ANOVA. Post hoc comparisons were made using Fisher's LSD tests. $^{**}P < 0.01$, $^{***}P < 0.001$, between-subjects effects of control versus anti-CD25 antibody, within the same CSC condition; $^{++}P < 0.01$, $^{+++}P < 0.001$, between-subjects effects of SHC versus CSC, within the same antibody condition. The number of independent data points (*N*) in each of the graphs and sample size (*n*) for each group are as follows: (*A*) *N* = 28; *M. vaccae*/SHC/control Ab, 7; *M. vaccae*/CSC/control Ab, 7; *M. vaccae*/SHC/anti-CD25 Ab, 6; *M. vaccae*/CSC/anti-CD25 Ab, 8. (*B*) *N* = 23; *M. vaccae*/SHC/control Ab, 5; *M. vaccae*/CSC/control Ab, 6; *M. vaccae*/SHC/anti-CD25 Ab, 5; *M. vaccae*/CSC/anti-CD25 Ab, 7. (*C–G*) *N* = 29–31; *M. vaccae*/SHC/control Ab, 8; *M. vaccae*/CSC/control Ab, 7; *M. vaccae*/SHC/anti-CD25 Ab, (*C*, *E*, and *G*) 8, (*D*) 6, (*F*) 7; *M. vaccae*/CSC/anti-CD25 Ab, 8.

$P < 0.01$]. In addition, there was a main effect of CSC to reduce the IL-6/IL-10 ratio, due to the exaggerated release of IL-10 in these *M. vaccae*-immunized, CSC-exposed mice (Fig. 3*G*) [CSC, $F_{(1, 28)} = 31.8$, $P < 0.0001$]. Together, these data support the hypothesis that stress-protective effects of *M. vaccae* to prevent colitis and promote anxiolytic/fear-reducing responses are dependent on activation of Treg and an antiinflammatory bias, whereas the shift toward a more proactive emotional coping response to stress is not. Alternatively, brain mechanisms driving proactive emotional coping responses may already be established by the time of Treg depletion, and are not reversible or are reversed over a longer time frame. A diagrammatical illustration of the overall hypothetical model is presented in Fig. S6*M*.

Discussion

The results support the conclusion that immunization with a heat-killed preparation of *M. vaccae* increases resilience to stress-related pathologies in part through the induction of Treg and an antiinflammatory bias. Immunization with *M. vaccae* decreased submissive behavioral displays, as well as flight and avoiding behaviors, during an initial encounter with a dominant male aggressor. Following exposure to the chronic psychosocial stressor for 19 d, mice immunized with *M. vaccae*, but not vehicle-immunized mice, responded with decreased anxiety- or fear-like behaviors when tested on the EPM. These behavioral responses were associated with altered gene expression in serotonergic systems previously implicated in stress resilience and changes in microglial density in brain structures implicated in control of fear expression. Immunization with *M. vaccae* also prevented stress-induced spontaneous colitis and stress-induced exaggeration of chemically induced colitis, a model of IBD. The effects of immunization with *M. vaccae* on anxiety- or fear-like behaviors in stressed mice, as well as its effects on stress-induced exaggeration of spontaneous colitis, were prevented by depletion of Treg. In contrast, there were no effects of Treg depletion on *M. vaccae*-induced changes in behavioral responses to a dominant aggressor, suggesting multiple different mechanisms through which immunization with *M. vaccae* alters stress-related behavior. Together, these data are consistent with the hypothesis that immunization with *M. vaccae* can prevent stress-induced exaggeration of colonic inflammation and downstream effects on anxiety- and fear-like behavior. As such, immunization with heat-killed preparations of immunoregulatory bacteria may have utility in prevention of anxiety and affective disorders and their medical comorbidity, including exaggerated autoimmunity (11, 59) and exaggerated symptoms of IBD (12, 11, 59).

When challenged with a dominant aggressor 1–2 wk following the final immunization with *M. vaccae*, mice showed a robust decrease in submissive upright posture and decreases in reactive behavioral coping responses, such as flight and avoiding behaviors, relative to vehicle-immunized controls. Previous studies have shown that a reactive emotional coping strategy during social defeat, as measured by a short latency to display submissive postures, predicts vulnerability to subsequent development of anxiety- and depressive-like behavioral responses (25, 27, 60, 61) and that inflammatory factors within the CNS drive the vulnerability to depressive-like behavioral responses in individuals with reactive coping responses (32). Thus, the decreased submissive behaviors in *M. vaccae*-immunized mice are consistent with a stress-resilient behavioral phenotype. The mechanisms underlying the shift in behavioral strategy during psychosocial stress are not clear, but do not seem to depend on Treg. Of potential importance, *M. vaccae*-immunized mice had altered *tph2* and *slc6a4* mRNA expression specifically in the dorsal parts of the rostral to midrostrocaudal dorsal raphe nucleus, a subregion of the dorsal raphe nucleus that has previously been implicated in stress resilience (62). The effects of immunization on *tph2* mRNA expression were observed in both SHC and CSC mice and were therefore independent of the stress-induced differences in peripheral inflammation.

Factors that are known to influence individual variability in stress coping behaviors during psychosocial stress include endocrine factors, such as testosterone, or increased sympathetic reactivity, which are both associated with a more proactive behavioral coping strategy (27). Interestingly, the microbiome generally (63) and the probiotic *Lactobacillus reuteri* specifically have been shown to increase testosterone in mice, effects that could be mimicked by interfering with IL-17A signaling, a proinflammatory cytokine (64). Immunoregulatory microbes act on regulatory dendritic cells, which in turn bias T-cell differentiation toward Treg and away from IL-17–producing T helper 17 (Th17) cells (65, 66). Testosterone in turn increases serotonin transporter mRNA expression and binding in rats and humans in brain regions innervated by the rostral dorsal raphe nucleus

(67, 68), an effect consistent with our finding that immunization with *M. vaccae* prevented stress-induced decreases in *slc6a4* mRNA expression specifically in the rostral dorsal raphe nucleus. In addition, testosterone increases the neuronal firing rates of serotonergic neurons in the dorsal raphe nucleus (69). The effects of testosterone on serotonin transporter mRNA, binding, and serotonergic neuronal firing are thought to be dependent on aromatization of testosterone to 17β-estradiol (68, 69); 17β-estradiol induces anxiolytic effects on the EPM, in association with increases in *tph2* mRNA expression, specifically in the dorsal part of the dorsal raphe nucleus (70), as observed in our studies. Thus, immunoregulatory microbes can increase plasma testosterone concentrations, and testosterone or its metabolite, 17β-estradiol, replicates the effects of *M. vaccae* immunization on behavioral coping strategies during social defeat, performance on the EPM, and *slc6a4* and *tph2* mRNA expression in the dorsal raphe nucleus. Future studies should explore these potential endocrine mechanisms, as well as their interactions with the microbiome–gut–brain and behavior axis.

The effects of *M. vaccae* immunization to decrease submissive behavioral displays and decrease flight and avoiding behaviors were long-lasting, evident at least 1–2 wk following the final immunization. Meanwhile, the effects of *M. vaccae* immunization to induce anxiolytic responses (measured on day 19) and prevent CSC-induced exaggeration of colitis (measured on day 20) were observed 4–5 wk following the final immunization. As demonstrated using anti-CD25 antibody experiments, the effects of *M. vaccae* immunization to induce anxiolytic responses and prevent stress-induced exaggeration of colitis appear to be dependent on Treg. This timeline is consistent with previous studies demonstrating a long half-life of Treg in C57BL/6 mice, estimated to be 27 d (71). Long-lasting protection following immunization with the same heat-killed preparation of *M. vaccae* has been observed in a murine model of allergic airway inflammation, where protective effects last up to 12 wk (21). Persistent effects of *M. vaccae* in this model may depend in part on Treg derived from memory T cells (72), as mice are repeatedly exposed to the allergen antigen, ovalbumin. It is likely, however, that repeated immunization would be necessary in adults to induce persistent immunoregulatory effects.

The stress-protective effects of *M. vaccae* immunization appear to be independent of the changes in the diversity or community structure of the gut microbiota. Consistent with previous studies, exposure of mice to chronic psychosocial stress resulted in decreased diversity of gut microbial communities, as measured by α-diversity, and altered gut microbial community structure. Stress-induced changes in gut microbial communities were driven by expansion of *Helicobacter* spp., which have been shown to induce colitis in hosts with impaired immunoregulation, such as IL-10$^{-/-}$ mice, through induction of IFN-γ and IL-12 (73, 74). Importantly, preinoculation with immunoregulatory *L. reuteri* and *Lactobacillus paracasei* reduced intestinal inflammation in *Helicobacter hepaticus*-challenged mice, despite failing to alter the quantity of *H. hepaticus* in cocolonized mice (75). *L. reuteri* primes dendritic cells to drive the development of Treg, through interactions with the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) (65). Our results parallel these previous studies, in that *Helicobacter* spp. relative abundance was correlated with colitis scores in vehicle-treated mice, whereas *M. vaccae*-immunized CSC mice, characterized by abrogated release of IFN-γ and IL-6, together with exaggerated release of IL-10 from freshly isolated mesenteric lymph node cells stimulated in vitro, were protected against both development of spontaneous colitis and aggravation of chemically induced colitis in a murine model of IBD.

The stress-protective effects of *M. vaccae* immunization also appear to be independent of the changes in the functionality of the HPA axis. Glucocorticoid hormones, acting at glucocorticoid receptors, have been shown to induce expansion of *Helicobacter* spp. (18). These findings are consistent with other studies showing that glucocorticoid hormones decrease IgA (which normally inhibits bacterial adherence to intestinal epithelial cells), increase bacterial adherence to the intestinal epithelium over twofold, and increase bacterial translocation to mesenteric lymph nodes (17). Mice exposed to the CSC procedure experience a profound activation of the HPA axis, with persistent adrenal hypertrophy that is evident within 24 h and persists through days 2, 7, 14, and 20 of the CSC procedure, exaggerated ACTH and corticosterone release to novel stressors, and in vitro adrenal insufficiency, as measured by decreased sensitivity to ACTH (26). Glucocorticoid insensitivity of effector immune cells, as indicated by glucocorticoid insensitivity of lipopolysaccharide-stimulated splenocytes and anti-CD3–stimulated T cells, may contribute to the exaggerated inflammatory responses in CSC mice (26). Immunization with *M. vaccae* had no effect on the CSC-induced adrenal hypertrophy or the in vitro adrenal insensitivity, suggesting that, although HPA axis changes may contribute to stress-induced exaggerations of inflammation, they do not mediate the protective effects of *M. vaccae* immunization.

As previously demonstrated for allergic airway inflammation (22), the beneficial effects of *M. vaccae* on colitis and measures of anxiety appear to be dependent on induction of Treg and enhanced immunoregulation. The effects of CSC exposure on colitis and anxiety may be linked, as DSS-induced colitis (76–78), or chronic gastrointestinal inflammation induced by *Trichuris muris* infection, is sufficient to induce anxiety-like behavioral responses. This effect may be mediated in part by afferent signaling of the vagus nerve, as prior vagotomy prevents the anxiogenic effects of DSS-induced colitis (77), although this is not the case for *T. muris* infection (79). Bacteria belonging to the phylum Actinobacteria, specifically *M. vaccae* (80), and probiotics including *Bifidobacterium breve 1205* (81), *Bifidobacterium longum 1714* (81), and *B. longum NCC3001*, have been shown to decrease anxiety-like behaviors in mice. A number of probiotics belonging to the phylum Firmicutes also have been shown to decrease anxiety-like behaviors in mice, including *Lactobacillus helveticus ROO52* (82) and *Lactobacillus rhamnosus* (*JB-1*) (83). Anxiolytic effects of *B. longum 1714* (81) and *L. rhamnosus* (*JB-1*) (83) are prevented by vagotomy, suggesting that signaling via the vagus nerve may be particularly important in the anxiolytic effects of some probiotics administered via the mucosal route. In our study, immunization with *M. vaccae* reduced spontaneous colitis and stress-induced exaggeration of chemically induced colitis and, in CSC mice, induced anxiolytic effects, effects that may involve both vagus-dependent and vagus-independent mechanisms.

In conclusion, these data suggest that exposure to environmental microorganisms, administration of probiotics with immunoregulatory actions, or immunoregulation-promoting immunizations with heat-killed preparations of these organisms or antigens derived from these organisms may confer health benefits, including mental health benefits in subjects with stress-related psychiatric disorders, such as PTSD and major depression, at least partly through prevention of stress-induced intestinal inflammation. From a broader perspective, immunoregulatory approaches such as that demonstrated here may prove useful in both prevention and treatment of stress-related psychiatric disorders, such as anxiety and affective disorders, in which inadequate immunoregulation, resulting in chronic, low-grade inflammation, is a risk factor. Although not specifically addressed here, immunoregulatory approaches may also prove useful in prevention of neurodevelopmental and other somatic and neuropsychiatric disorders in which elevated inflammation contributes to disease vulnerability (84). Lack of exposure to "old friends" through reduced contact with healthy soils and healthy animals in modern

urban settings may partly explain the increased rates of immune-mediated diseases, including psychiatric disorders, in these settings. Restoring exposure to these old friends, through immunization or other routes, may decrease inflammation-associated disease vulnerability in modern urban societies.

Materials and Methods

For detailed materials and methods, see *SI Materials and Methods*. All experimental protocols were approved by the Committee on Animal Health and Care of the government of Oberpfalz, Germany, and were performed according to international guidelines on the ethical use of animals.

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1. Sonnenburg ED, Sonnenburg JL (2014) Starving our microbial self: The deleterious consequences of a diet deficient in microbiota-accessible carbohydrates. *Cell Metab* 20(5):779–786.
2. von Hertzen L, et al. (2015) Helsinki alert of biodiversity and health. *Ann Med* 47(3): 218–225.
3. Atherton JC, Blaser MJ (2009) Coadaptation of *Helicobacter pylori* and humans: Ancient history, modern implications. *J Clin Invest* 119(9):2475–2487.
4. Rook GA, Raison CL, Lowry CA (2014) Microbial 'old friends', immunoregulation and socioeconomic status. *Clin Exp Immunol* 177(1):1–12.
5. Ohnmacht C, et al. (2015) The microbiota regulates type 2 immunity through RORγt$^+$ T cells. *Science* 349(6251):989–993.
6. Sefik E, et al. (2015) Individual intestinal symbionts induce a distinct population of RORγ^+ regulatory T cells. *Science* 349(6251):993–997.
7. Eraly SA, et al.; Marine Resiliency Study Team (2014) Assessment of plasma C-reactive protein as a biomarker of posttraumatic stress disorder risk. *JAMA Psychiatry* 71(4): 423–431.
8. Hodes GE, et al. (2014) Individual differences in the peripheral immune system promote resilience versus susceptibility to social stress. *Proc Natl Acad Sci USA* 111(45):16136–16141.
9. Sommershof A, et al. (2009) Substantial reduction of naïve and regulatory T cells following traumatic stress. *Brain Behav Immun* 23(8):1117–1124.
10. Lindqvist D, et al. (2014) Proinflammatory milieu in combat-related PTSD is independent of depression and early life stress. *Brain Behav Immun* 42:81–88.
11. O'Donovan A, et al. (2015) Elevated risk for autoimmune disorders in Iraq and Afghanistan veterans with posttraumatic stress disorder. *Biol Psychiatry* 77(4):365–374.
12. Cámara RJ, Gander ML, Begré S, von Känel R; Swiss Inflammatory Bowel Disease Cohort Study Group (2011) Post-traumatic stress in Crohn's disease and its association with disease activity. *Frontline Gastroenterol* 2(1):2–9.
13. Breen MS, et al. (2015) Gene networks specific for innate immunity define post-traumatic stress disorder. *Mol Psychiatry* 20(12):1538–1545.
14. Bailey MT, et al. (2011) Exposure to a social stressor alters the structure of the intestinal microbiota: Implications for stressor-induced immunomodulation. *Brain Behav Immun* 25(3):397–407.
15. Bailey MT (2014) Influence of stressor-induced nervous system activation on the intestinal microbiota and the importance for immunomodulation. *Adv Exp Med Biol* 817:255–276.
16. Cryan JF, Dinan TG (2012) Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* 13(10):701–712.
17. Alverdy J, Aoys E (1991) The effect of glucocorticoid administration on bacterial translocation. Evidence for an acquired mucosal immunodeficient state. *Ann Surg* 214(6):719–723.
18. Guo G, et al. (2009) Psychological stress enhances the colonization of the stomach by *Helicobacter pylori* in the BALB/c mouse. *Stress* 12(6):478–485.
19. Sheil B, et al. (2004) Is the mucosal route of administration essential for probiotic function? Subcutaneous administration is associated with attenuation of murine colitis and arthritis. *Gut* 53(5):694–700.
20. Laudanno O, Vasconcelos L, Catalana J, Cesolari J (2006) Anti-inflammatory effect of bioflora probiotic administered orally or subcutaneously with live or dead bacteria. *Dig Dis Sci* 51(12):2180–2183.
21. Zuany-Amorim C, et al. (2002) Long-term protective and antigen-specific effect of heat-killed *Mycobacterium vaccae* in a murine model of allergic pulmonary inflammation. *J Immunol* 169(3):1492–1499.
22. Zuany-Amorim C, et al. (2002) Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. *Nat Med* 8(6):625–629.
23. Le Bert N, Chain BM, Rook G, Noursadeghi M (2011) DC priming by *M. vaccae* inhibits Th2 responses in contrast to specific TLR2 priming and is associated with selective activation of the CREB pathway. *PLoS One* 6(4):e18346.
24. Bryant RA, Marosszeky JE, Crooks J, Baguley I, Gurka J (2000) Coping style and post-traumatic stress disorder following severe traumatic brain injury. *Brain Inj* 14(2): 175–180.
25. Wood SK, Walker HE, Valentino RJ, Bhatnagar S (2010) Individual differences in reactivity to social stress predict susceptibility and resilience to a depressive phenotype: Role of corticotropin-releasing factor. *Endocrinology* 151(4):1795–1805.
26. Langgartner D, Füchsl AM, Uschold-Schmidt N, Slattery DA, Reber SO (2015) Chronic subordinate colony housing paradigm: A mouse model to characterize the consequences of insufficient glucocorticoid signaling. *Front Psychiatry* 6:18.
27. Koolhaas JM, et al. (1999) Coping styles in animals: Current status in behavior and stress-physiology. *Neurosci Biobehav Rev* 23(7):925–935.
28. Reber SO, et al. (2007) Adrenal insufficiency and colonic inflammation after a novel chronic psycho-social stress paradigm in mice: Implications and mechanisms. *Endocrinology* 148(2):670–682.
29. Greenwood BN, et al. (2005) Wheel running alters serotonin (5-HT) transporter, 5-HT1A, 5-HT1B, and alpha 1b-adrenergic receptor mRNA in the rat raphe nuclei. *Biol Psychiatry* 57(5):559–568.
30. Greenwood BN, et al. (2003) Freewheel running prevents learned helplessness/behavioral depression: Role of dorsal raphe serotonergic neurons. *J Neurosci* 23(7): 2889–2898.
31. Loughridge AB, Greenwood BN, Day HE, McQueen MB, Fleshner M (2013) Microarray analyses reveal novel targets of exercise-induced stress resistance in the dorsal raphe nucleus. *Front Behav Neurosci* 7:37.
32. Wood SK, et al. (2015) Inflammatory factors mediate vulnerability to a social stress-induced depressive-like phenotype in passive coping rats. *Biol Psychiatry* 78(1):38–48.
33. Erny D, et al. (2015) Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* 18(7):965–977.
34. Wang Y, Kasper LH (2014) The role of microbiome in central nervous system disorders. *Brain Behav Immun* 38:1–12.
35. Ahmed Z, et al. (2007) Actin-binding proteins coronin-1a and IBA-1 are effective microglial markers for immunohistochemistry. *J Histochem Cytochem* 55(7):687–700.
36. Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S (1996) A novel gene *iba1* in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem Biophys Res Commun* 224(3):855–862.
37. Vinet J, et al. (2012) Neuroprotective function for ramified microglia in hippocampal excitotoxicity. *J Neuroinflammation* 9:27.
38. Sierra-Mercado D, Padilla-Coreano N, Quirk GJ (2011) Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology* 36(2): 529–538.
39. Maier SF (2015) Behavioral control blunts reactions to contemporaneous and future adverse events: Medial prefrontal cortex plasticity and a corticostriatal network. *Neurobiol Stress* 1:12–22.
40. Zhang H, Sparks JB, Karyala SV, Settlage R, Luo XM (2015) Host adaptive immunity alters gut microbiota. *ISME J* 9(3):770–781.
41. Mandal S, et al. (2015) Analysis of composition of microbiomes: A novel method for studying microbial composition. *Microb Ecol Health Dis* 26:27663.
42. Bassett SA, et al. (2015) Changes in composition of caecal microbiota associated with increased colon inflammation in interleukin-10 gene-deficient mice inoculated with *Enterococcus* species. *Nutrients* 7(3):1798–1816.
43. Lin P, et al. (2014) HLA-B27 and human β2-microglobulin affect the gut microbiota of transgenic rats. *PLoS One* 9(8):e105684.
44. Belzer C, et al. (2014) Dynamics of the microbiota in response to host infection. *PLoS One* 9(7):e95534.
45. Graff LA, Walker JR, Bernstein CN (2009) Depression and anxiety in inflammatory bowel disease: A review of comorbidity and management. *Inflamm Bowel Dis* 15(7): 1105–1118.
46. Round JL, Mazmanian SK (2010) Inducible Foxp3$^+$ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci USA* 107(27): 12204–12209.
47. Sokol H, et al. (2008) *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci USA* 105(43):16731–16736.
48. Zuo H, et al. (2016) Plasma biomarkers of inflammation, the kynurenine pathway, and risks of all-cause, cancer, and cardiovascular disease mortality: The Hordaland Health Study. *Am J Epidemiol* 183(4):249–258.
49. Lin HM, et al. (2010) Metabolomic analysis identifies inflammatory and non-inflammatory metabolic effects of genetic modification in a mouse model of Crohn's disease. *J Proteome Res* 9(4):1965–1975.
50. Freier E, et al. (2010) Decrease of CD4(+)FOXP3(+) T regulatory cells in the peripheral blood of human subjects undergoing a mental stressor. *Psychoneuroendocrinology* 35(5):663–673.
51. Wu W, et al. (2014) Prolactin mediates psychological stress-induced dysfunction of regulatory T cells to facilitate intestinal inflammation. *Gut* 63(12):1883–1892.
52. Kim SJ, et al. (2012) CD4$^+$CD25$^+$ regulatory T cell depletion modulates anxiety and depression-like behaviors in mice. *PLoS One* 7(7):e42054.
53. Grosse L, et al. (2016) Circulating cytotoxic T cells and natural killer cells as potential predictors for antidepressant response in melancholic depression. Restoration of T regulatory cell populations after antidepressant therapy. *Psychopharmacology (Berl)* 233(9):1679–1688.
54. Morath J, et al. (2014) The effect of trauma-focused therapy on the altered T cell distribution in individuals with PTSD: Evidence from a randomized controlled trial. *J Psychiatr Res* 54:1–10.

55. Long TT, Nakazawa S, Onizuka S, Huaman MC, Kanbara H (2003) Influence of $CD4^+CD25^+$ T cells on *Plasmodium berghei* NK65 infection in BALB/c mice. *Int J Parasitol* 33(2):175–183.
56. Taylor MD, et al. (2005) Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. *J Immunol* 174(8): 4924–4933.
57. Hollis JH, Evans AK, Bruce KP, Lightman SL, Lowry CA (2006) Lipopolysaccharide has indomethacin-sensitive actions on Fos expression in topographically organized subpopulations of serotonergic neurons. *Brain Behav Immun* 20(6):569–577.
58. Lowry CA, et al. (2007) Identification of an immune-responsive mesolimbocortical serotonergic system: Potential role in regulation of emotional behavior. *Neuroscience* 146(2): 756–772.
59. Eaton WW, Pedersen MG, Nielsen PR, Mortensen PB (2010) Autoimmune diseases, bipolar disorder, and non-affective psychosis. *Bipolar Disord* 12(6):638–646.
60. Veenema AH, Torner L, Blume A, Beiderbeck DI, Neumann ID (2007) Low inborn anxiety correlates with high intermale aggression: Link to ACTH response and neuronal activation of the hypothalamic paraventricular nucleus. *Horm Behav* 51(1): 11–19.
61. Wood SK, Bhatnagar S (2015) Resilience to the effects of social stress: Evidence from clinical and preclinical studies on the role of coping strategies. *Neurobiol Stress* 1: 164–173.
62. Greenwood BN, Fleshner M (2011) Exercise, stress resistance, and central serotonergic systems. *Exerc Sport Sci Rev* 39(3):140–149.
63. Markle JG, et al. (2013) Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* 339(6123):1084–1088.
64. Poutahidis T, et al. (2014) Probiotic microbes sustain youthful serum testosterone levels and testicular size in aging mice. *PLoS One* 9(1):e84877.
65. Smits HH, et al. (2005) Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J Allergy Clin Immunol* 115(6):1260–1267.
66. Arnold IC, Hitzler I, Müller A (2012) The immunomodulatory properties of *Helicobacter pylori* confer protection against allergic and chronic inflammatory disorders. *Front Cell Infect Microbiol* 2:10.
67. Kranz GS, et al. (2015) High-dose testosterone treatment increases serotonin transporter binding in transgender people. *Biol Psychiatry* 78(8):525–533.
68. McQueen JK, Wilson H, Sumner BE, Fink G (1999) Serotonin transporter (SERT) mRNA and binding site densities in male rat brain affected by sex steroids. *Brain Res Mol Brain Res* 63(2):241–247.
69. Robichaud M, Debonnel G (2005) Oestrogen and testosterone modulate the firing activity of dorsal raphe nucleus serotonergic neurones in both male and female rats. *J Neuroendocrinol* 17(3):179–185.
70. Donner N, Handa RJ (2009) Estrogen receptor beta regulates the expression of tryptophan-hydroxylase 2 mRNA within serotonergic neurons of the rat dorsal raphe nuclei. *Neuroscience* 163(2):705–718.
71. Dépis F, Kwon HK, Mathis D, Benoist C (2016) Unstable $FoxP3^+$ T regulatory cells in NZW mice. *Proc Natl Acad Sci USA* 113(5):1345–1350.
72. Vukmanovic-Stejic M, et al. (2006) Human $CD4^+$ $CD25^{hi}$ $Foxp3^+$ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest* 116(9):2423–2433.
73. Kullberg MC, et al. (1998) *Helicobacter hepaticus* triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. *Infect Immun* 66(11):5157–5166.
74. Burich A, et al. (2001) *Helicobacter*-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. *Am J Physiol Gastrointest Liver Physiol* 281(3):G764–G778.
75. Peña JA, et al. (2005) Probiotic *Lactobacillus* spp. diminish *Helicobacter hepaticus*-induced inflammatory bowel disease in interleukin-10-deficient mice. *Infect Immun* 73(2):912–920.
76. Hassan AM, et al. (2014) Repeated predictable stress causes resilience against colitis-induced behavioral changes in mice. *Front Behav Neurosci* 8:386.
77. Bercik P, et al. (2011) The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterol Motil* 23(12):1132–1139.
78. Painsipp E, Herzog H, Sperk G, Holzer P (2011) Sex-dependent control of murine emotional-affective behaviour in health and colitis by peptide YY and neuropeptide Y. *Br J Pharmacol* 163(6):1302–1314.
79. Bercik P, et al. (2010) Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. *Gastroenterology* 139(6):2102–2112.e1.
80. Matthews DM, Jenks SM (2013) Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice. *Behav Processes* 96:27–35.
81. Savignac HM, Kiely B, Dinan TG, Cryan JF (2014) Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterol Motil* 26(11):1615–1627.
82. Ohland CL, et al. (2013) Effects of *Lactobacillus helveticus* on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. *Psychoneuroendocrinology* 38(9):1738–1747.
83. Bravo JA, et al. (2011) Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* 108(38):16050–16055.
84. Choi GB, et al. (2016) The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science* 351(6276):933–939.
85. Spear LP (2000) The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev* 24(4):417–463.
86. Singewald GM, Nguyen NK, Neumann ID, Singewald N, Reber SO (2009) Effect of chronic psychosocial stress-induced by subordinate colony (CSC) housing on brain neuronal activity patterns in mice. *Stress* 12(1):58–69.
87. Reber SO, Neumann ID (2008) Defensive behavioral strategies and enhanced state anxiety during chronic subordinate colony housing are accompanied by reduced hypothalamic vasopressin, but not oxytocin, expression. *Ann N Y Acad Sci* 1148: 184–195.
88. Uschold-Schmidt N, Nyuyki KD, Füchsl AM, Neumann ID, Reber SO (2012) Chronic psychosocial stress results in sensitization of the HPA axis to acute heterotypic stressors despite a reduction of adrenal in vitro ACTH responsiveness. *Psychoneuroendocrinology* 37(10):1676–1687.
89. Donner NC, et al. (2012) Elevated *tph2* mRNA expression in a rat model of chronic anxiety. *Depress Anxiety* 29(4):307–319.
90. Gardner KL, Hale MW, Lightman SL, Plotsky PM, Lowry CA (2009) Adverse early life experience and social stress during adulthood interact to increase serotonin transporter mRNA expression. *Brain Res* 1305:47–63.
91. Franklin KBJ, Paxinos G (2005) *The Mouse Brain in Stereotaxic Coordinates* (Academic, San Diego).
92. Beynon SB, Walker FR (2012) Microglial activation in the injured and healthy brain: What are we really talking about? Practical and theoretical issues associated with the measurement of changes in microglial morphology. *Neuroscience* 225:162–171.
93. Lechner A, et al. (2007) Protective immunity and delayed type hypersensitivity reaction are uncoupled in experimental *Leishmania major* infection of CCR6-negative mice. *Microbes Infect* 9(3):291–299.
94. Caporaso JG, et al. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* 108(Suppl 1):4516–4522.
95. Caporaso JG, et al. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 6(8):1621–1624.
96. Caporaso JG, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7(5):335–336.
97. Navas-Molina JA, et al. (2013) Advancing our understanding of the human microbiome using QIIME. *Methods Enzymol* 531:371–444.
98. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26(19):2460–2461.
99. DeSantis TZ, et al. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72(7):5069–5072.
100. McDonald D, et al. (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* 6(3):610–618.
101. Caporaso JG, et al. (2010) PyNAST: A flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26(2):266–267.
102. Price MN, Dehal PS, Arkin AP (2010) FastTree 2—Approximately maximum-likelihood trees for large alignments. *PLoS One* 5(3):e9490.
103. Grubbs FE (1969) Procedures for detecting outlying observations in samples. *Technometrics* 11(1):1–21.
104. Verma SP, Díaz-González L, Rosales-Rivera M, Quiroz-Ruiz A (2014) Comparative performance of four single extreme outlier discordancy tests from Monte Carlo simulations. *ScientificWorldJournal* 2014:746451.
105. Rousselet GA, Pernet CR (2012) Improving standards in brain-behavior correlation analyses. *Front Hum Neurosci* 6:119.
106. Faith DP (1992) Conservation evaluation and phylogenetic diversity. *Biol Conserv* 61(1):1–10.
107. Lozupone C, Knight R (2005) UniFrac: A new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71(12):8228–8235.
108. Vázquez-Baeza Y, Pirrung M, Gonzalez A, Knight R (2013) EMPeror: A tool for visualizing high-throughput microbial community data. *Gigascience* 2(1):16.
109. Hamady M, Lozupone C, Knight R (2010) Fast UniFrac: Facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *ISME J* 4(1):17–27.
110. Lozupone CA, Hamady M, Kelley ST, Knight R (2007) Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Appl Environ Microbiol* 73(5):1576–1585.
111. Martin-Fernandez JA, Barcelo-Vidal C, Pawlowsky-Glahn V (2003) Dealing with zeros and missing values in compositional data sets using nonparametric imputation. *Math Geol* 35(3):253–278.
112. Pawlowsky-Glahn V, Egozcue JJ, Tolosana-Delgado R (2007) *Lecture Notes on Compositional Data Analysis* (Universitat de Girona, Girona, Spain).
113. Krishnaswamy S, et al. (2014) Conditional density-based analysis of T cell signaling in single-cell data. *Science* 346(6213):1250689.
114. Egozcue JJ, Pawlowsky-Glahn V, Mateu-Figueras G, Barcelo-Vidal C (2003) Isometric logratio transformations for compositional data analysis. *Math Geol* 35(3):279–300.
115. American Psychiatric Association (2013) *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, Arlington, VA), 5th Ed.
116. Korte SM, De Boer SF (2003) A robust animal model of state anxiety: Fear-potentiated behaviour in the elevated plus-maze. *Eur J Pharmacol* 463(1–3):163–175.
117. Slattery DA, et al. (2012) Behavioural consequences of two chronic psychosocial stress paradigms: Anxiety without depression. *Psychoneuroendocrinology* 37(5):702–714.
118. Boscarino JA (1997) Diseases among men 20 years after exposure to severe stress: Implications for clinical research and medical care. *Psychosom Med* 59(6):605–614.
119. Husarewycz MN, El-Gabalawy R, Logsetty S, Sareen J (2014) The association between number and type of traumatic life experiences and physical conditions in a nationally representative sample. *Gen Hosp Psychiatry* 36(1):26–32.
120. Reber SO (2012) Stress and animal models of inflammatory bowel disease—An update on the role of the hypothalamo-pituitary-adrenal axis. *Psychoneuroendocrinology* 37(1):1–19.
121. Reber SO, et al. (2011) Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis. *Brain Behav Immun* 25(6):1153–1161.
122. Boscarino JA, Chang J (1999) Higher abnormal leukocyte and lymphocyte counts 20 years after exposure to severe stress: Research and clinical implications. *Psychosom Med* 61(3):378–386.

123. Reber SO, Obermeier F, Straub RH, Veenema AH, Neumann ID (2008) Aggravation of DSS-induced colitis after chronic subordinate colony (CSC) housing is partially mediated by adrenal mechanisms. *Stress* 11(3):225–234.
124. Daskalakis NP, Lehrner A, Yehuda R (2013) Endocrine aspects of post-traumatic stress disorder and implications for diagnosis and treatment. *Endocrinol Metab Clin North Am* 42(3):503–513.
125. Veenema AH, Reber SO, Selch S, Obermeier F, Neumann ID (2008) Early life stress enhances the vulnerability to chronic psychosocial stress and experimental colitis in adult mice. *Endocrinology* 149(6):2727–2736.
126. Yehuda R, Golier JA, Kaufman S (2005) Circadian rhythm of salivary cortisol in Holocaust survivors with and without PTSD. *Am J Psychiatry* 162(5):998–1000.
127. Yehuda R, Golier JA, Halligan SL, Meaney M, Bierer LM (2004) The ACTH response to dexamethasone in PTSD. *Am J Psychiatry* 161(8):1397–1403.
128. Füchsl AM, Langgartner D, Reber SO (2013) Mechanisms underlying the increased plasma ACTH levels in chronic psychosocially stressed male mice. *PLoS One* 8(12): e84161.
129. de Kloet CS, et al. (2006) Assessment of HPA-axis function in posttraumatic stress disorder: Pharmacological and non-pharmacological challenge tests, a review. *J Psychiatr Res* 40(6):550–567.
130. Wieck A, Grassi-Oliveira R, Hartmann do Prado C, Teixeira AL, Bauer ME (2014) Neuroimmunoendocrine interactions in post-traumatic stress disorder: Focus on long-term implications of childhood maltreatment. *Neuroimmunomodulation* 21(2–3):145–151.
131. Zhou J, et al. (2014) Dysregulation in microRNA expression is associated with alterations in immune functions in combat veterans with post-traumatic stress disorder. *PLoS One* 9(4):e94075.
132. Schmidt D, et al. (2010) Chronic psychosocial stress promotes systemic immune activation and the development of inflammatory Th cell responses. *Brain Behav Immun* 24(7):1097–1104.
133. Belzung C, Lemoine M (2011) Criteria of validity for animal models of psychiatric disorders: Focus on anxiety disorders and depression. *Biol Mood Anxiety Disord* 1:9.
134. Herman JL (1992) Complex PTSD: A syndrome in survivors of prolonged and repeated trauma. *J Trauma Stress* 5(3):377–391.