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Subcutaneous *Mycobacterium vaccae* ameliorates the effects of early life adversity alone or in combination with chronic stress during adulthood in male and female mice

Giulia Mazzari [a], Christopher A. Lowry [b,c,d,e], Dominik Langgartner [a], Stefan O. Reber [a,*]

[a] Laboratory for Molecular Psychosomatics, Department of Psychosomatic Medicine and Psychotherapy, Ulm University Medical Center, 89081, Ulm, Germany
[b] Department of Integrative Physiology, Department of Psychology and Neuroscience, Center for Neuroscience and Center for Microbial Exploration, University of Colorado Boulder, Boulder, CO, 80309, USA
[c] Department of Physical Medicine and Rehabilitation and Center for Neuroscience, University of Colorado Anschutz Medical Campus, Aurora, CO, 80045, USA
[d] Veterans Health Administration, Rocky Mountain Mental Illness Research Education and Clinical Center (MIRECC), The Rocky Mountain Regional Veterans Affairs Medical Center (RMRVAMC), Aurora, CO, 80045, USA
[e] Military and Veteran Microbiome: Consortium for Research and Education (MVM-CoRE), Aurora, CO, 80045, USA

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ABSTRACT

Chronic psychosocial stress is a burden of modern society and poses a clear risk factor for a plethora of somatic and affective disorders, of which most are associated with an activated immune status and chronic low-grade inflammation. Preclinical and clinical studies further suggest that a failure in immunoregulation promotes an over-reaction of the inflammatory stress response and, thus, predisposes an individual to the development of stress-related disorders. Therefore, all genetic (i.e., sex) and environmental (i.e., early life adversity; ELA) factors facilitating an adult's inflammatory stress response are likely to increase their stress vulnerability.

In the present study we investigated whether repeated subcutaneous (s.c.) administrations with a heat-killed preparation of *Mycobacterium vaccae* (*M. vaccae*; National Collection of Type Cultures (NCTC) 11659), an abundant soil saprophyte with immunoregulatory properties, are protective against negative behavioral, immunological and physiological consequences of ELA alone or of ELA followed by chronic psychosocial stress during adulthood (CAS) in male and female mice. ELA was induced by the maternal separation (MS) paradigm, CAS was induced by 19 days of chronic subordinate colony housing (CSC) in males and by a 7-week exposure to the social instability paradigm (SIP) in females.

Our data indicate that ELA effects in both sexes, although relatively mild, were to a great extent prevented by subsequent s.c. *M. vaccae* administrations. Moreover, although the use of different paradigms for males and females impedes a direct comparison, male mice seemed to be more susceptible to CAS than females, with only females benefitting slightly from the stress protective effects of s.c. *M. vaccae* administrations when given prior to CAS alone. Finally, our data support the hypothesis that female mice are more vulnerable to the additive effects of ELA and CAS than male mice and that s.c. *M. vaccae* administrations subsequent to ELA but prior to CAS are protective in both sexes.

Taken together and considering the limitation that CAS in males and females was induced by different paradigms, our findings are consistent with the hypotheses that murine stress vulnerability during different phases of life is strongly sex dependent and that developing immunoregulatory approaches, such as repeated s.c. administrations with immunoregulatory microorganisms, have potential for prevention/treatment of stress-related disorders.

* Corresponding author. Laboratory for Molecular Psychosomatics, Department of Psychosomatic Medicine and Psychotherapy, Ulm University Medical Center, Albert-Einstein-Allee 11, 89081, Ulm, Germany.
E-mail address: stefan.reber@uni-ulm.de (S.O. Reber).

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Abbreviations

ACTH	adrenocorticotropic hormone
BBS	borate-buffered saline
BM	bone marrow
CAS	chronic adult stress
CORT	corticosterone
CSC	chronic subordinate colony housing
CV	coefficient of variation
DSS	dextran sulfate sodium
ELA	early life adversity
FBS	fetal bovine serum
FCS	fetal calf serum
GC	glucocorticoid
G-CSF	granulocyte colony-stimulating factor
GHC	group-housed controls
HBSS	Hanks' Balanced Salt solution
IBD	inflammatory bowel disease
IL	interleukin
KC	keratinocyte chemoattractant
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MO-MDSC	monocyte-like-myeloid-derived suppressor cells
MS	maternal separation
Mvac	*Mycobacterium vaccae* NCTC 11659
MWU	Mann-Whitney *U* test
NCTC	National Collection of Type Cultures
NO	novel object
noMS	no maternal separation
OD	optical density
OF	open-field
PMN-MDSC	polymorphonucler myeloid-derived suppressor cells
PND	postnatal day
PTSD	posttraumatic stress disorder
RPMI-1640	Roswell Park Memorial Institute Medium-1640
s.c.	subcutaneous
SHC	single-housed controls
SIP	social instability paradigm
SPAT	social preference/avoidance test
SPF	specific-pathogen free
TNF	tumor necrosis factor
Treg	regulatory T cells

1. Introduction

Chronic psychosocial stress is a major burden of modern life and poses a clear risk factor for many somatic and affective disorders, including inflammatory bowel disease (IBD) (Bernstein, 2010; Bernstein et al., 2010; Langgartner et al., 2019) and posttraumatic stress disorder (PTSD) (Yehuda and Seckl, 2011). Although the underlying mechanisms are still largely unknown, most stress-associated somatic and affective disorders are accompanied by an activated immune status and chronic low-grade inflammation (Lindqvist et al., 2014, 2017; Pace et al., 2006, 2012). Given that typical stress-associated disorders, as for instance PTSD, are further associated with decreased numbers of regulatory T cells (Tregs) (Sommershof et al., 2009), these findings suggest that a failure in immunoregulation promotes an over-reaction of the inflammatory response to trauma and, thus, predisposes an individual to the development of stress-related disorders in general, and PTSD in particular. Importantly, results from both preclinical and clinical studies are consistent with this hypothesis (Eraly et al., 2014; Hodes et al., 2014; Khandaker et al., 2014; Pervanidou et al., 2007; Schultebraucks et al., 2021; Voigt et al., 2022).

Noteworthy, adverse consequences of psychological stress during adulthood vary strongly between individuals, with some being more resilient than others (Kolassa et al., 2010a, 2010b; Wilker et al., 2013). Of interest in this context, when compared to men, women are at a two to three fold higher risk of developing PTSD, which is in line with the overall increased prevalence of stress-related psychiatric disorders in women vs. men (Kessler, 1994; Kessler et al., 2005; McLean et al., 2011). Given the above described link between an over-reacting inflammatory response and the development of stress-associated disorders, all genetic and environmental factors facilitating an adult's immune (re-)activity are, therefore, likely to increase their stress vulnerability (Brown et al., 2021).

One such environmental factor might be early life adversity (ELA). This hypothesis is consistent with evidence indicating that psychological trauma has particularly adverse consequences if it occurs in a cumulative manner (Kolassa et al., 2010a, 2010b). Moreover, child maltreatment is well-known to cause chronic low-grade inflammation, characterized by increased levels of proinflammatory cytokines and C-reactive protein, fibrinogen, and white blood cells (Baumeister et al., 2016; Danese et al., 2007; Surtees et al., 2003). Most interestingly, psychosocial stress has been shown repeatedly to activate peripheral inflammatory pathways (Rohleder, 2014; Steptoe et al., 2007), and to do so more robustly in people with histories of early life abuse and/or neglect (Carpenter et al., 2010; Pace et al., 2006), who are also at significantly heightened risk for PTSD development in response to trauma exposure in adult life (Pace and Heim, 2011).

Consistent with the aforementioned role of immune activation in development of stress-related disorders, we have previously shown that immunoregulation induced by repeated subcutaneous (s.c) immunizations with a heat-killed preparation of *Mycobacterium vaccae* (*M. vaccae*; National Collection of Type Cultures (NCTC) 11659), an abundant soil saprophyte with immunoregulatory properties, was able to promote active stress coping and to prevent stress-induced anxiety, spontaneous colitis as well as aggravation of dextran sulfate sodium (DSS)-induced colitis, in a chronic adult stress (CAS) model of PTSD in male mice (Amoroso et al., 2020; Reber et al., 2016a). In extension of these findings, we recently showed that *M. vaccae* also prevents stress-induced exaggeration of anxiety-like defensive behavioral responses, when administered repeatedly via the s.c. route during chronic psychosocial stressor exposure of male mice (Amoroso et al., 2020). Moreover, intragastric (i.g.) *M. vaccae* was able to protect against stress-induced glucocorticoid (GC) resistance (Langgartner et al., 2023) and, when administered via the non-invasive intranasal (i.n.) route prior to or during stressor exposure, ameliorated or totally prevented stress-induced aggravation of DSS-induced colitis in male mice (Amoroso et al., 2019). Of note, many clinical and preclinical studies (Avitsur et al., 2002; Bellingrath et al., 2013; Engler et al., 2005; Miller and Raison, 2016; Raison et al., 2006; Raison and Miller, 2003; Stark et al., 2001) support the hypothesis that stress-associated inflammation is promoted at least in part via development of GC resistance, defined as a state of reduced sensitivity to the anti-inflammatory action of GCs, in certain immune cell subpopulations (Miller and Raison, 2016; Raison and Miller, 2003) amongst which $CD11b^{+}$ myeloid cells in general (Avitsur et al., 2002; Engler et al., 2004; Foertsch et al., 2017; Stark et al., 2001), and polymorphonuclear (PMN)-myeloid-derived suppressor cells (MDSCs) in particular (Kempter et al., 2023) seem to play a critical role. To date, the stress protective effects of *M. vaccae* NCTC 11659 in rodent models have only been investigated in males, which represents an important knowledge gap.

The aim of the present study was to investigate in male and female mice whether repeated s.c. administrations of *M. vaccae* following early life stress (ELA; i.e., 1st hit) but prior to CAS (i.e., 2nd hit) are able to

reverse and/or protect against additive negative consequences of multiple life stressors (Fig. 1). ELA was induced by 3 h/day of maternal separation (MS) during the first two weeks of life (Heim and Nemeroff, 2001; Plotsky and Meaney, 1993; Veenema, 2009). CAS was induced by 19 days of chronic subordinate colony housing (CSC) in males (Reber et al., 2007, 2016a) and by a 7-week exposure to the social instability paradigm (SIP) in females (Schmidt et al., 2010b; Sterlemann et al., 2008; Yohn et al., 2019).

2. Materials and Methods

2.1. Animals

Breeding male and female C57BL/6N mice obtained from Charles River (Sulzfeld, Germany) were used to generate in-house bred C57BL/6N offspring used as experimental mice (=cohort 1). Male CD-1 mice (30–35 g; Charles River, Sulzfeld, Germany) were used as dominant aggressors during CSC exposure and as stimulus mice in the social preference/avoidance test (SPAT) of male experimental mice of cohort 1 and female mice of cohort 2 (=additional set of female mice to verify that female mice do not show social preference towards a male stimulus mouse in the social preference/avoidance test (SPAT); see also Section 2.2). Therefore, female C57BL/6N non-pregnant breeding mice were used as stimulus mice in the SPAT of female experimental mice of cohort 1. All mice were kept in standard polycarbonate mouse cages (16 cm width × 22 cm length × 14 cm height) under standard specific-pathogen free (SPF) laboratory conditions (12-h light–dark cycle, lights on: 0600 a.m. (winter time), 0700 a.m. (summer time); 22 °C, 60% humidity) and had free access to tap water and standard mouse diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government and performed according to national and international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering. The research described here was conducted in compliance with the ARRIVE Guidelines for Reporting Animal Research (Percie du Sert et al., 2020).

2.2. Experimental procedures

The experimental timelines are shown in Fig. 1A (male mice) and Fig. 1B (female mice). In detail, pregnant females were observed daily and, if litters were found before 02:00 p.m., the same day was assigned to postnatal day (PND) 0, if litters were found after 02:00 p.m., the following day was assigned to PND 0. At PND 1, litters were randomly assigned to the MS or "no maternal separation" (noMS) group and exposed to MS or noMS (see Section 2.4 for details) daily until PND 14. Afterwards, all litters were housed together with their dams until weaning at PND 21. Following weaning, experimental male MS and noMS mice were housed according to their treatment and sex in groups of 3–4 per cage for 1–2 weeks before receiving repeated (3x) weekly s.c. administrations with sterile borate-buffered saline (BBS) vehicle or *M. vaccae* NCTC 11659 (0.1 mg/0.1 mL per s.c. injection) in sterile BBS. One to two weeks after the last administration, all experimental mice were exposed to the first round of behavioral testing (open field/novel object (OF/NO1) test followed by SPAT1 on consecutive days). The day after SPAT1, experimental male mice were exposed to the CSC paradigm (3 weeks), a preclinical murine model for PTSD (Amoroso et al., 2019, 2020; Langgartner et al., 2015; Reber et al., 2007; Reber and Neumann, 2008; Reber et al., 2016), while experimental female mice were exposed to the SIP (7 weeks) (Schmidt et al., 2008; Sterlemann et al., 2008; Yohn et al., 2019). Controls were kept as single-housed (SHC; males) or group-housed (GHC; females). Litter effects were controlled by assuring that every group was composed by no more than $N = 3$ pups from the same litter. For group assignment see *Supplementary Figure* (SupFig.) *2*. On Days 19/20 of CSC and on Days 48/49 of SIP, all experimental mice were exposed to the second round of behavioral testing (OF/NO2 test followed by the SPAT2 on consecutive days), before being euthanized the respective following day between 07:00 a.m. and 10:00 a.m. for the assessment of physiological and immunological parameters described

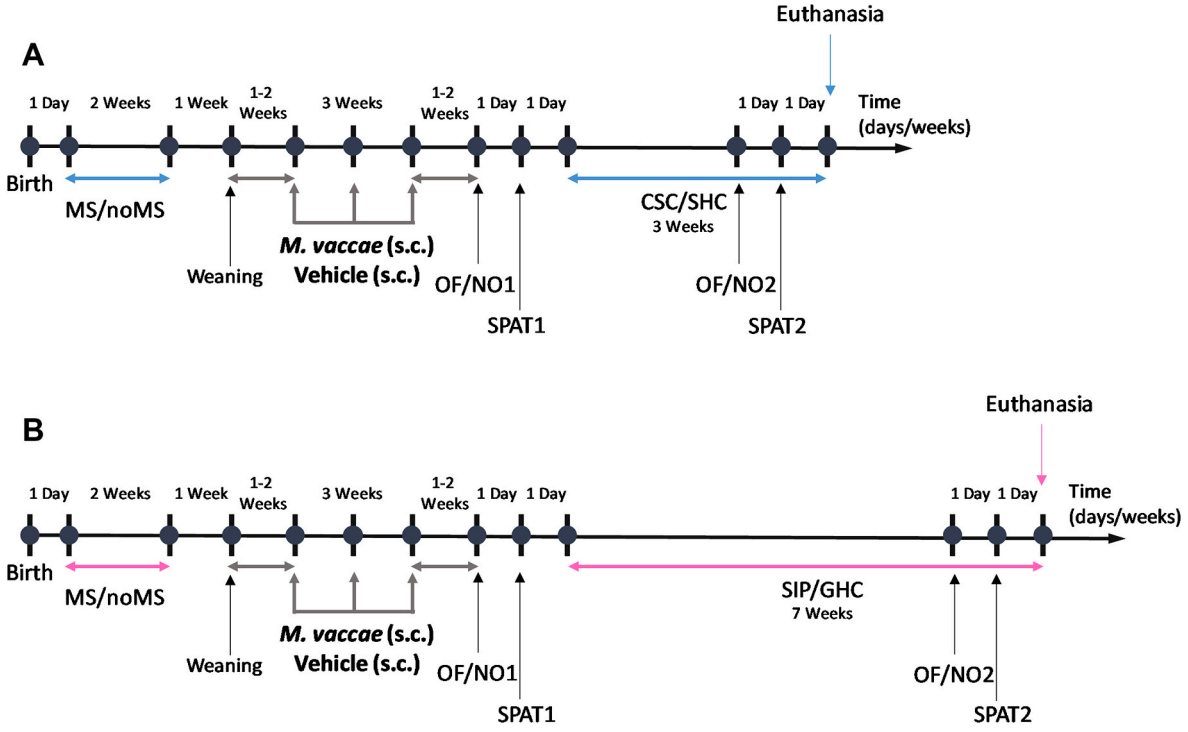

Fig. 1. Experimental timelines. Schematic illustration of the experimental timelines for (A) male and (B) female mice (for details, see *Materials & Methods*). ELA was induced by MS in both sexes; CAS was induced by CSC in male and SIP in female mice. Abbreviations: CSC, chronic subordinate colony housing; GHC, group-housed control; MS, maternal separation; *M. vaccae*, *Mycobacterium vaccae;* noMS, no maternal separation; OF/NO test, open field/novel object test; PNDs, postnatal days; SHC, single-housed control; SIP, social instability paradigm; SPAT, social preference/avoidance test; s.c., subcutaneous.

below.

In order to confirm that female stimulus mice are required for female experimental mice to show social preference in the SPAT, a second set (=cohort 2) of female noMS-exposed BBS-treated mice ($N = 35$) was exposed to a male stimulus mouse during SPAT one day following OF/NO testing.

2.3. Administration of M. vaccae and BBS

Experimental mice received repeated s.c. immunizations with either 0.1 mg whole heat-killed *M. vaccae* suspension [10 mg/mL solution; strain NCTC 11659, batch ENG 1, provided by BioElpida (Lyon, France) diluted to 1 mg/mL in 100 µL sterile BBS] or injections of 100 µL of the vehicle (BBS) using 21-gauge needles once per week for 3 consecutive weeks, respectively. Of note, this is the same dose that our group has used previously for s.c (Amoroso et al., 2020; Reber et al., 2016b). and i.g (Langgartner et al., 2023). administrations of *M. vaccae* in mice.

2.4. Maternal separation

Maternal separation was performed as previously described (Veenema, 2009; Veenema et al., 2006; Wigger and Neumann, 1999). Briefly, pups were separated daily from their dams for 3 h (0800–1100 during wintertime; 0900–1200 during summertime) between PND 1 and 14. First, dams were removed from the maternity cage and placed into separate individual cages. Pups were then transferred as a whole litter into a small box and placed onto a heating pad (30–33 °C) in an adjacent room. After the 3 h separation period, the pups were returned to the home cage, followed by reunion with the dam. Unseparated litters (noMS) served as controls and were left undisturbed, except for daily handling. Bedding was not changed for the entire duration of the MS paradigm.

2.5. Chronic subordinate colony housing (CSC) procedure

The CSC paradigm was conducted as previously described (Amoroso et al., 2019, 2020; Langgartner et al., 2015; Reber et al., 2007; Reber and Neumann, 2008). For further details, see *SI Materials and Methods*.

2.6. Social instability paradigm (SIP)

The SIP was performed as previously described (Schmidt et al., 2008; Sterlemann et al., 2008; Yohn et al., 2019), with minor modifications. Briefly, the cage composition of experimental mice was changed every third day over a period of seven consecutive weeks. To minimize the chance of repeatedly encountering the same cage mates, group rotation was randomized using GraphPad "Assign subjects to groups" (GraphPad Software, San Diego, CA) online version. Body weight and possible bite wounds were assessed every time the cage composition was changed. Respective GHCs were housed in groups of 3–4 mice for 7 weeks.

2.7. Open-field/novel object (OF/NO) test

To assess the effects of *M. vaccae* and/or stress on anxiety-related behavior, the OF/NO test was conducted as previously described (Amoroso et al., 2020; Foertsch et al., 2017; Langgartner et al., 2015). For further details, see *SI Materials and Methods*.

2.8. Social preference/avoidance test (SPAT)

To assess the effects of *M. vaccae* and/or stress on social preference/avoidance, the SPAT was conducted as described previously (Slattery et al., 2012). For further details, see *SI Materials and Methods*.

2.9. Trunk blood sampling

Trunk blood was collected as described recently (Amoroso et al., 2019, 2020). For further details, see *SI Materials and Methods*.

2.10. Assessment of adrenals, pituitary and spleen weight

After decapitation, pituitary and spleen of each mouse were removed, pruned of fat and weighed. Spleens were subsequently stored in ice-cold Hanks' Balanced Salt solution (HBSS; Sigma-Aldrich) until being used for the isolation of splenocytes. Adrenal glands were removed, pruned of fat and stored in ice-cold Dulbecco's Modified Eagle Medium (DMEM/F-12, Life Technologies, Inc, Grand Island, NY, USA; supplemented with 0.1% bovine serum albumin (BSA; Biomol, Hamburg, Germany)), until being weighed. Of note, adrenal weights of three male mice (noMS-Mvac-CSC: $N = 1$; MS-Veh-SHC: $N = 1$; MS-Mvac-SHC: $N = 1$) are not included in the analysis because of experimental issues.

2.11. Multiplex cytokine measurement

Plasma cytokine concentrations were measured using a Bio-Plex Pro cytokine assay (BioRad, Munich, Germany) as described previously (Langgartner et al., 2018b). For further details, see *SI Materials and Methods*. Two female mice were excluded because of a problem that occurred during the measurement (noMS-Veh-GHC: $N = 2$).

2.12. Assessment of the severity of bite wounds

The severity of bite wounds in CSC mice was assessed using a bite score previously established by our group (Foertsch et al., 2017). For further details, see *SI Materials and Methods*.

2.13. Splenocytes isolation

The isolation of splenocytes for the glucocorticoid (GC) sensitivity assay was done as previously described (Foertsch et al., 2017). For further details, see *SI Materials and Methods*.

2.14. Functional in vitro glucocorticoid sensitivity assay of isolated splenocytes

Functional splenic *in vitro* GC resistance was assessed as previously described (Foertsch et al., 2017, 2020; Kempter et al., 2023). For further details, see *SI Materials and Methods*. Two male and two female mice were excluded from the assay because of pipetting mistakes (noMS-Mvac-CSC: $N = 1$; MS-Veh-SHC: $N = 1$; MS-Mvac-GHC: $N = 1$; MS-Mvac-SIP: $N = 1$).

2.15. Flow cytometric analysis of total splenocytes

Splenocytes were incubated with fluorescently labelled antibodies (CD11b, APC-eFluor 780, BD Bioscience, Franklin Lakes, NJ, USA; Ly6G, V450, BD Bioscience, Franklin Lakes, NJ, USA; Ly6C, APC, BD Bioscience, Franklin Lakes, NJ, USA) at 4 °C for 30 min. Following antibody incubation, cells were washed twice with FACS buffer (PBS, 10% FCS, 0.1% NaN3) before being fixed using the Foxp3/Transcription Factor Staining Buffer Set Kit (eBioscience, Carlsbad, CA, USA). Afterwards, samples were transferred into FACS buffer and analyzed using a LSR II flow cytometer (BD Bioscience) and Flow Jo Version 10 (Tree Star, Ashland, OR, USA). A total number of 50,000 events was analyzed for each sample.

2.16. Statistics

For statistical analysis and graphical illustrations, GraphPad Prism

(version 9.3.1, GraphPad Software, LCC) was used. Kolmogorov-Smirnov test with Lilliefors' correction was employed to test for normal distribution. Extreme outliers in normally distributed data sets were identified by Grubbs test and excluded from further analysis (Grubbs, 1969) (Males: "OF1: distance moved", MS-Mvac: $N = 1$; Females: "Relative adrenal weight", MS-Mvac-GHC: $N = 1$). Normally distributed data sets were analyzed by parametric statistics, i.e., paired *t*-test (two dependent groups, which differ in one factor), two-way ANOVA (two factors, two or more independent samples) and three-way ANOVA (more than two factors, two or more independent samples). Non-normally distributed data sets were analyzed by non-parametric statistics, i.e., Mann-Whitney *U* test (MWU, one factor, two independent samples) and Wilcoxon test (one factor, two dependent samples). All statistical tests comparing more than two samples were followed by *post hoc* analysis using Bonferroni pairwise comparison (parametric statistics) or Dunn's multiple comparison (non-parametric statistics), when a significant main effect was found. Normally distributed data are presented as bar plots (mean +SEM) including individual values. Non-normally distributed data are presented as box plots (median (thick line); mean (plus sign); 25th and 75th percentiles; minimum and maximum values, outliers) including individual values. The level of significance was set at $P \leq 0.05$. For summary of statistical effects and the 95% confidence interval (CI) of difference (diff), see *Supplementary Table* (SupTab.) 1.

3. Results

Results are always presented in chronological order, starting with the effects of ELA (induced by MS in both sexes), followed by the effects of CAS (induced by CSC in males and SIP in females) and closing with the interaction effects between both ELA and CAS. Stress protective effects of *M. vaccae* are reported for each section. All statistical effects are reported in SupTab 1.

3.1. Effects of ELA and M. vaccae on general (PND 48) and social anxiety-related behavior (PND 49)

In **males**, statistical analysis revealed no differences between the groups in the overall distance travelled during OF1 exposure (Fig. 2C). In contrast, MS-Mvac mice spent more time in the inner zone during OF1 exposure compared to respective noMS mice (Fig. 2D). During NO1 exposure MS vs. noMS mice in the Veh but not Mvac group spent more time in the contact zone (Fig. 2E). However, the time spent in direct contact with the empty cage during SPAT1, which also represents a novel object for the mice, was comparable between all groups (Fig. 2F). During SPAT1 (Fig. 2F) exposure, all groups except the MS-Veh group spent more time in direct contact with the social (i.e., male) vs. empty cage. Time spent in direct contact with the social stimulus was increased in MS-Mvac vs. MS-Veh mice (Fig. 2F).

In **females**, locomotion (Fig. 2K), time spent in the inner zone during OF1 testing (Fig. 2L) and time in contact zone during NO1 exposure (Fig. 2M) were comparable between all groups. During SPAT1 exposure, all groups spent more time in the direct contact with social (i.e., female) vs. empty cage condition (Fig. 2N).

Of note, to to confirm that female stimulus mice are required for female experimental mice to show social preference in the SPAT, a second set (=cohort 2) of female noMS-exposed BBS-treated mice ($N = 35$) was exposed to a male stimulus mouse in the SPAT and compared to a subset of noMS-exposed BBS-treated female experimental mice of cohort 1, which were exposed to a female stimulus mouse ($N = 19$; see SupFig. 1A, B, E, F for schematic representation and track visualization). Statistical analysis revealed that female test mice in presence of a male social stimulus did not differ in time spent in corners between the empty and social condition (SupFig. 1C), while spending less time in direct contact with the social-compared to the empty cage condition (SupFig. 1D). In contrast, a female social stimulus decreased time spent in the corners (SupFig. 1G) and increased time spent in direct contact with the social stimulus (SupFig. 1H), both compared with respective empty cage condition.

3.2. Effects of ELA, M. vaccae and CAS on general (PND 68/98) and social anxiety-related behavior (PND 69/99)

In **males**, overall distance travelled during OF2 exposure was comparable between all groups (Fig. 2G). No effects were further revealed for the time spent in the inner zone during OF2 and the time in the contact zone during NO2 exposure (Fig. 2H and I), and time in direct contact with the empty cage during SPAT2 exposure was comparable between all groups (Fig. 2J). However, time in the direct contact with the social (i.e., male) vs. empty cage during SPAT2 exposure was increased significantly (Fig. 2J) in all SHC but not in the respective CSC groups. In addition, noMS-Mvac-CSC and MS-Mvac-CSC mice spent less time in direct contact with the social cage (i.e., male) compared with respective SHC mice.

In **females**, statistical analysis revealed that distance travelled (Fig. 2O) was neither affected by MS, *M. vaccae,* nor SIP. Time spent in the inner zone during OF2 exposure was significantly reduced in MS-Mvac-GHC (Fig. 2P) compared with respective Veh-treated mice, and by trend vs. respective SIP-exposed mice. Time spent in the contact zone during NO exposure was also decreased in GHC vs. SIP mice in the MS-Mvac group (Fig. 2Q). All groups further spent more time in direct contact with the social (i.e., female) vs. empty cage during SPAT exposure (Fig. 2R).

3.3. Effects of ELA, M. vaccae and CAS on HPA axis activity

In **males,** statistical analysis revealed that relative pituitary weight was higher in CSC vs. SHC mice only in the noMS-Mvac and MS-Mvac groups (Fig. 3A). Although relative adrenal weight (Fig. 3B) was higher in all CSC groups compared with respective SHC groups, this effect was most pronounced in the MS-Mvac-CSC group.

In **females,** statistical analysis did not reveal any differences in relative pituitary (Fig. 3C) and adrenal weight (Fig. 3D) between the groups.

3.4. Effects of ELA, M. vaccae and CAS on the spleen

In **males**, relative spleen weight (Fig. 4A) was increased in CSC vs. SHC mice of all groups, with this effect being more pronounced in the MS-Mvac compared with the respective MS-Veh and noMS-Mvac group, but by trend less pronounced in the noMS-Veh-CSC vs. MS-Veh-CSC group. The bite score (Fig. 4B) of MS-Veh-CSC mice was lower compared with both respective noMS-Veh-CSC mice and MS-Mvac-CSC mice, but the latter was by trend higher than the respective noMS-Mvac-CSC mice. Basal *in vitro* cell viability of isolated splenocytes (Fig. 4C) was increased in MS-CSC mice compared with respective MS-SHC mice and in MS-Mvac-CSC mice compared with respective noMS-Mvac-CSC mice. Moreover, LPS increased (Fig. 4D) the *in vitro* cell viability of isolated splenocytes in all experimental groups compared with respective basal values, with the effects being more pronounced in CSC vs. SHC mice of the noMS-Mvac, MS-Veh and MS-Mvac groups. Absolute delta cell viability (LPS minus basal conditions; Fig. 4D) was increased in CSC mice of the noMS-Mvac, MS-Veh and MS-Mvac group compared with respective SHC mice. Finally, relative delta cell viability (LPS-basal; 0 μM CORT set to 100%) of isolated and *in vitro* cultured splenocytes in the presence of 0.1 μM CORT was significantly lower in all groups (Fig. 4E) compared with the respective 0 μM CORT condition. In addition, delta cell viability in CSC vs. SHC mice was increased in all groups except the MS-Veh group, with MS-Mvac-CSC mice showing a higher delta cell viability compared with respective Veh mice.

In **females**, relative spleen weight (Fig. 4F) was decreased in MS-Veh-GHC mice compared with respective noMS-Veh-GHC mice, as

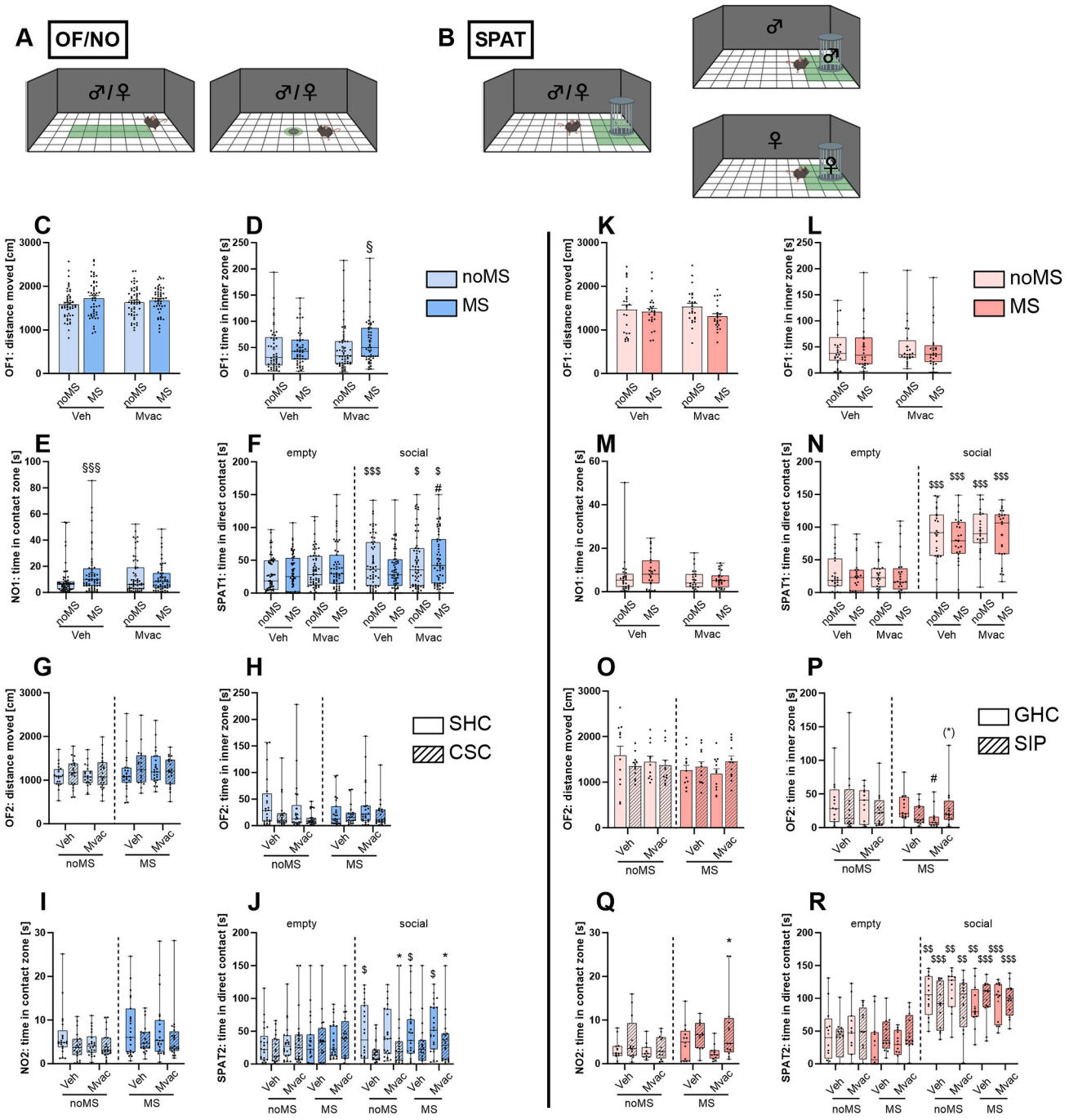

Fig. 2. Effects of ELA, *M. vaccae* and CAS on general and social anxiety-like behaviors. Schematic illustration of the test area and the experimental setting for the (A) OF/NO test and (B) SPAT. (C–J) Behavioral tests in males: (C) OF1 - distance moved, (D) OF1 - time in inner zone, (E) NO1 - time in contact zone, (F) SPAT1 - time in direct contact, (G) OF2 - distance moved, (H) OF2 - time in inner zone, (I) NO2 - time in contact zone, (J) SPAT2 - time in direct contact. (K–R) Behavioral tests in females: (K) OF1 - distance moved, (L) OF1 - time in inner zone, (M) NO1 - time in contact zone, (N) SPAT1 - time in direct contact, (O) OF2 - distance moved, (P) OF2 - time in inner zone, (Q) NO2 - time in contact zone, (R) SPAT2 - time in direct contact. ELA was induced by MS in both sexes; CAS was induced by CSC in male and SIP in female mice. Parametric data are presented as mean +SEM including individual values. Non-parametric data are presented as box-plots including individual values, with solid line representing the median and plus sign representing the mean for each data-set. Lower boxes indicate 25th, upper boxes 75th percentile; minimum (lower error bar), maximum (upper error bar) as well as possible outliers (closed circles beyond the percentiles) are also shown. (*) $p \leq 0.08$, *$p \leq 0.05$ versus respective SHC/GHC condition; #$p \leq 0.05$ versus respective Mvac condition; §$p \leq 0.05$; §§§$p \leq 0.001$ versus respective noMS condition; \$ $p \leq 0.05$, \$\$ $p \leq 0.01$, \$\$\$ $p \leq 0.001$ versus respective empty cage condition. For detailed statistical effects see Results section and SupTab. 1. Abbreviations: CSC, chronic subordinate colony housing; GHC, group-housed control; MS, maternal separation; Mvac, *M. vaccae*, *Mycobacterium vaccae;* noMS, no maternal separation; OF/NO test, open field/novel object test; SHC, single-housed control; SIP, social instability paradigm; SPAT, social preference/avoidance test; Veh, vehicle (borate-buffered saline).

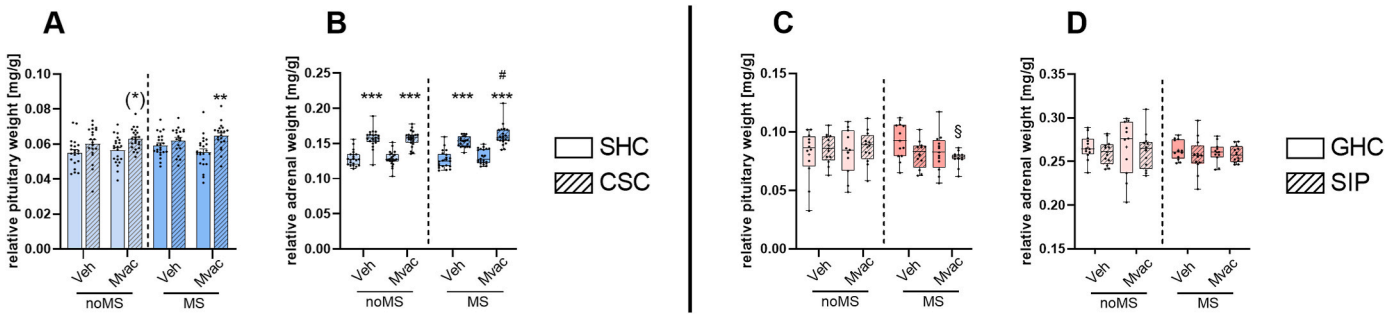

Fig. 3. Effects of ELA, *M. vaccae* and CAS on the HPA axis. (A, B) Males: (A) relative pituitary weight and (B) relative adrenal weight. (C, D) Females: (C) relative pituitary weight and (D) relative adrenal weight. ELA was induced by MS in both sexes; CAS was induced by CSC in male and SIP in female mice. Parametric data are presented as mean +SEM including individual values. Non-parametric data are presented as box-plots including individual values, with solid line representing the median and plus sign representing the mean for each data set. Lower boxes indicate 25th, upper boxes 75th percentile; minimum (lower error bar), maximum (upper error bar) as well as possible outliers (closed circles beyond the percentiles) are also shown. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ versus respective SHC/GHC condition; $\#p \leq 0.05$ versus respective Mvac condition; $\S p \leq 0.05$ versus respective noMS condition. For detailed statistical effects see Results section and SupTab.1. Abbreviations: CSC, chronic subordinate colony housing; GHC, group-housed control; MS, maternal separation; Mvac, *M. vaccae*, *Mycobacterium vaccae;* noMS, no maternal separation; SHC, single-housed control; SIP, social instability paradigm; Veh, vehicle (borate-buffered saline).

well as by trend in noMS-Veh-SIP vs. noMS-Veh-GHC and MS-Veh-GHC vs. MS-Mvac-GHC. Bite wounds were not detectable in any of the groups (Fig. 4G). Basal *in vitro* cell viability of isolated splenocytes (Fig. 4H) was significantly decreased in MS vs. noMS mice of the Mvac-GHC condition, and in MS-Veh-SIP vs. MS-Mvac-SIP mice. Moreover, LPS increased (Fig. 4H) the *in vitro* cell viability of isolated splenocytes in all experimental groups compared with respective basal values, with the effects being more pronounced in SIP vs. GHC mice of the MS-Veh mice, but interestingly decreased LPS-induced *in vitro* cell viability in MS-Mvac-SIP mice when compared with both respective MS-Veh-SIP mice and respective noMS-Mvac-SIP mice. Absolute delta cell viability (LPS minus basal conditions; Fig. 4I) was increased in MS-Veh-SIP mice compared to both respective MS-GHC-SIP mice and respective noMS-Veh-SIP mice, and by trend in MS-Mvac-GHC compared to the respective MS-Veh-GHC. Relative delta cell viability (LPS-basal; 0 μM CORT set to 100%) of isolated and *in vitro* cultured splenocytes in the presence of 0.1 μM CORT was significantly lower in all groups (Fig. 4J) compared with the respective 0 μM CORT condition.

3.5. Effects of ELA, M. vaccae and CAS on splenocytes subpopulations

In **males**, flow cytometry (see Fig. 5A for the gating scheme) revealed an increased percentage of splenic CD11b$^+$ myeloid cells in CSC vs. SHC mice in the noMS-Veh (Fig. 5B), noMS-Mvac and MS-Mvac group, as well as in MS-Veh-SHC mice compared with respective noMS-Veh-SHC mice and by trend in noMS-Mvac-CSC vs. noMS-Veh-CSC mice. In addition, MS-Veh-CSC mice showed lower percentages of CD11b$^+$ splenocytes when compared with respective MS-Mvac-CSC mice and noMS-Veh-CSC mice. With respect to splenic CD11b$^+$ myeloid subpopulations, the percentages of CD11b$^+$Ly6G$^+$Ly6C$^+$ PMN-MDSCs (Fig. 5D) and CD11b$^+$Ly6G$^+$Ly6C$^-$ neutrophils (Fig. 5E) cells were increased in CSC vs. SHC mice of the noMS-Veh (CD11b$^+$Ly6G$^+$Ly6C$^+$; CD11b$^+$Ly6G$^+$Ly6C$^-$), the noMS-Mvac (CD11b$^+$Ly6G$^+$Ly6C$^+$; CD11b$^+$Ly6G$^+$Ly6C$^-$) and the MS-Mvac (CD11b$^+$Ly6G$^+$Ly6C$^+$; CD11b$^+$Ly6G$^+$Ly6C$^-$) groups. Additionally, the percentages of CD11b$^+$Ly6G$^+$Ly6C$^+$ and CD11b$^+$Ly6G$^+$Ly6C$^-$ splenocytes were significantly increased in Mvac-CSC compared to respective Veh in the noMS and MS groups. The percentage of splenic CD11b$^+$Ly6G$^-$Ly6C$^+$ monocytes/monocyte-like (MO)-MDSCs was only by trend increased in noMS-Mvac-SHC vs. noMS-Veh-SHC mice (Fig. 5C).

In **females**, the percentages of splenic CD11b$^+$ cells (Fig. 5F) were increased in SIP vs. GHC mice in the noMS-Veh, noMS-Mvac and MS-Veh groups. Moreover, it was by trend decreased in MS-SIP-Mvac mice compared with respective Veh and noMS groups. Additionally, the percentages of CD11b$^+$Ly6G$^+$Ly6C$^+$ (Fig. 5H) and CD11b$^+$Ly6G$^+$Ly6C$^-$ (Fig. 5I) splenocyte subpopulations were increased in SIP vs. GHC mice of the noMS-Veh and of CD11b$^+$Ly6G+$^+$Ly6C$^+$ splenocytes also in the noMS-Mvac and MS-Mvac groups. The percentage of CD11b$^+$Ly6G$^+$Ly6C$^+$ cells was further lower in the MS-Mvac-SIP mice compared with respective Veh and noMS mice, while it was increased in MS-Mvac-GHC mice compared with respective Veh and noMS mice. The percentage of CD11b$^+$Ly6G$^-$Ly6C$^+$ splenocytes was not different between the groups (Fig. 5G).

3.6. Effects of ELA, M. vaccae and CAS on plasma cytokine concentrations

Statistical analysis revealed the following effects on cytokines (MWU, Fig. 6A-R).

In **males (**Fig. 6A–I), statistical analysis revealed a CSC-induced increase in plasma IL-1β (Fig. 6A), IL-4 (Fig. 6B), IL-6 (Fig. 6C), IL-10 (Fig. 6D), IL-17a (Fig. 6E), MCP-1 (Fig. 6H) and TNF (Fig. 6I) concentrations compared to SHC mice in all four experimental groups, while CSC vs. SHC mice showed increased plasma G-CSF (Fig. 6F) and KC (Fig. 6G) concentrations in all experimental groups except the noMS-Veh group. In addition, plasma IL-1β was increased in MS-Mvac-CSC vs. respective Veh mice (Fig. 6A), plasma IL-4 was lower in MS-Veh-SHC vs. respective noMS mice (Fig. 6B), plasma IL-6 was by trend lower in noMS-Mvac-SHC vs. respective Veh mice (Fig. 6C) and plasma MCP-1 was lower in noMS-Mvac-SHC mice vs. respective Veh mice (Fig. 6H), while it was increased in MS-Veh-CSC vs. respective noMS mice. Moreover, plasma KC was by trend lower in noMS-Mvac-SHC vs. respective Veh mice (Fig. 6G) and significantly higher in MS-Veh-CSC vs. respective noMS mice.

In **females**, statistical analysis revealed SIP-induced increases in the noMS-Veh-SIP group for IL-4 (Fig. 6K) and KC (Fig. 6P) compared to respective GHC mice. IL-10 was by trend lower in noMS-Mvac-GHC mice (Fig. 6M) compared with respective Veh mice. MS exposure further by trend or significantly increased plasma IL-17a in Mvac-GHC (Fig. 6N), G-CSF in Veh-SIP (Fig. 6O) and Mvac-GHC (Fig. 6O), KC in Veh-GHC (Fig. 6P) and Mvac-GHC (Fig. 6P), MCP-1 in Veh-GHC (Fig. 6Q) and Veh-SIP (Fig. 6Q) and TNF in Mvac-GHC (Fig. 6R) mice compared with respective noMS mice. IL-1β (Fig. 6J) and IL-6 (Fig. 6L) were below the respective detections limits of the Bio-Plex Pro cytokine assay.

4. Discussion

Psychosocial stress has been shown in clinical studies to activate peripheral inflammatory pathways (Rohleder, 2014; Steptoe et al., 2007), and to do so more robustly in people with histories of early life

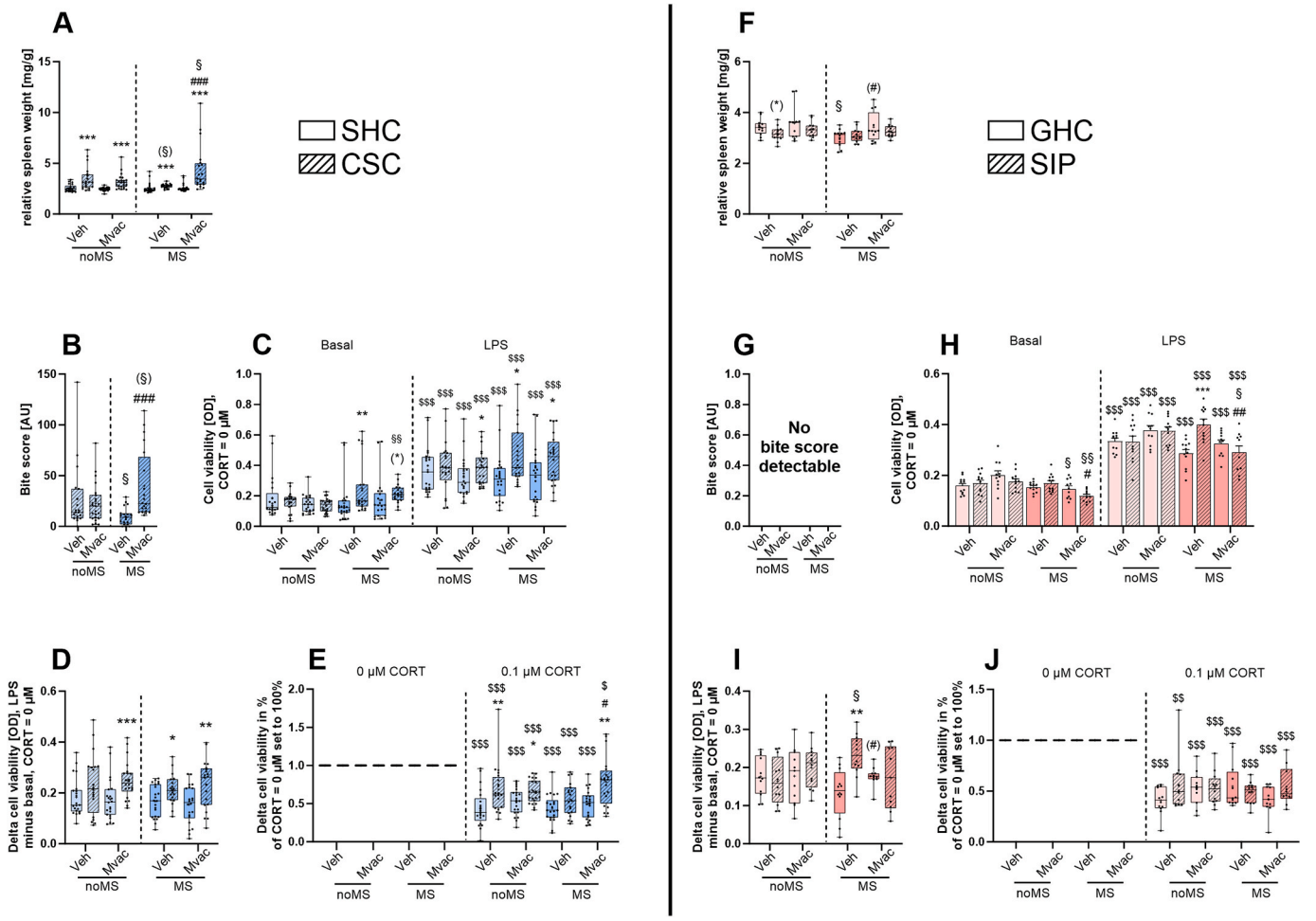

Fig. 4. Effects of ELA, *M. vaccae* and CAS on the spleen. (A–E) Males: (A) relative spleen weight, (B) bite score, (C) cell viability of isolated and *in vitro* stimulated splenocytes under basal and LPS-stimulated conditions in the absence of CORT, (D) delta cell viability (LPS-stimulated minus basal conditions) of isolated and *in vitro* stimulated splenocytes in the absence of CORT, (E) delta cell viability (LPS-stimulated minus basal conditions) of isolated and *in vitro* stimulated splenocytes in the presence of 0.1 µM of CORT (CORT = 0 µM set to 100%). (F–J) Females: (F) relative spleen weight, (G) bites core, (H) cell viability of isolated and *in vitro* stimulated splenocytes under basal and LPS-stimulated conditions in the absence of CORT, (I) delta cell viability (LPS-stimulated minus basal conditions) of isolated and *in vitro* stimulated splenocytes in the absence of CORT, (J) delta cell viability (LPS-stimulated minus basal conditions) of isolated and *in vitro* stimulated splenocytes in the presence of 0.1 µM of CORT (CORT = 0 µM set to 100%). ELA was induced by MS in both sexes; CAS was induced by CSC in male and SIP in female mice. Parametric data are presented as mean +SEM including individual values. Non-parametric data are presented as box-plots including individual values, with solid line representing the median and plus sign representing the mean for each data set. Lower boxes indicate 25th, upper boxes 75th percentile; minimum (lower error bar), maximum (upper error bar) as well as possible outliers (closed circles beyond the percentiles) are also shown. (*) $p \leq 0.08$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus respective SHC/GHC condition; (#) $p \leq 0.08$, #$p \leq 0.05$, ##$p \leq 0.01$, ###$p \leq 0.001$ versus respective Mvac condition; §$p \leq 0.05$, §§$p \leq 0.01$ versus respective noMS condition; $ $p \leq 0.05$, $$ $p \leq 0.01$, $$$ $p \leq 0.001$ versus respective basal/0 µM CORT condition. For detailed statistical effects see Results section and SupTab.1. Abbreviations: CORT, corticosterone; CSC, chronic subordinate colony housing; GHC, group-housed control; LPS, lipopolysaccharide; MS, maternal separation; Mvac, *M. vaccae*, *Mycobacterium vaccae;* noMS, no maternal separation; SHC, single-housed control; SIP, social instability paradigm; Veh, vehicle (borate-buffered saline).

abuse and/or neglect (Carpenter et al., 2010; Pace et al., 2006), who are also at significantly heightened risk for PTSD development in response to trauma exposure in adult life (Pace and Heim, 2011). Moreover, women are at increased risk to develop stress-related psychiatric disorders, including PTSD (Kessler, 1994; Kessler et al., 2005; McLean et al., 2011), and repeated administrations of immunomodulatory *M. vaccae* in male mice have been shown to be protective against the negative consequences of chronic psychosocial stress during adulthood, when administered either prior to or during stressor exposure (Amoroso et al., 2019, 2020, 2021; Reber et al., 2016b). Therefore, it was the main objective in this study to investigate in male and female mice whether repeated s.c. administrations of *M. vaccae* following ELA (i.e., 1st hit) but prior to CAS (i.e., 2nd hit; males: CSC; females: SIP) are able to reverse and/or protect against (additive) negative consequences of multiple life stressors. Our key findings were that: 1) ELA effects in both sexes, although relatively mild, were to a great extent prevented by subsequent s.c. *M. vaccae* administrations; 2) males seemed to be more susceptible to CAS than females, at least when neglecting that CAS in both sexes was induced by different psychosocial stress paradigms, with only females benefitting slightly from the stress protective effects of s.c. *M. vaccae* administrations prior to CAS alone; 3) additive effects of ELA and CAS seemed to be more pronounced in female relative to male mice and were prevented by s.c. *M. vaccae* administrations subsequent to ELA but prior to CAS. To the best of our knowledge, this is the first study to demonstrate that *M. vaccae* prevents adverse outcomes of stressor exposure in female mice.

4.1. *Effects on general and social anxiety-related behavior*

Maternal separation has been reported to increase anxiety-related behavior and stress reactivity in male mice (Gracia-Rubio et al., 2016; Romeo et al., 2003; Veenema et al., 2008) and male rats (Huot et al.,

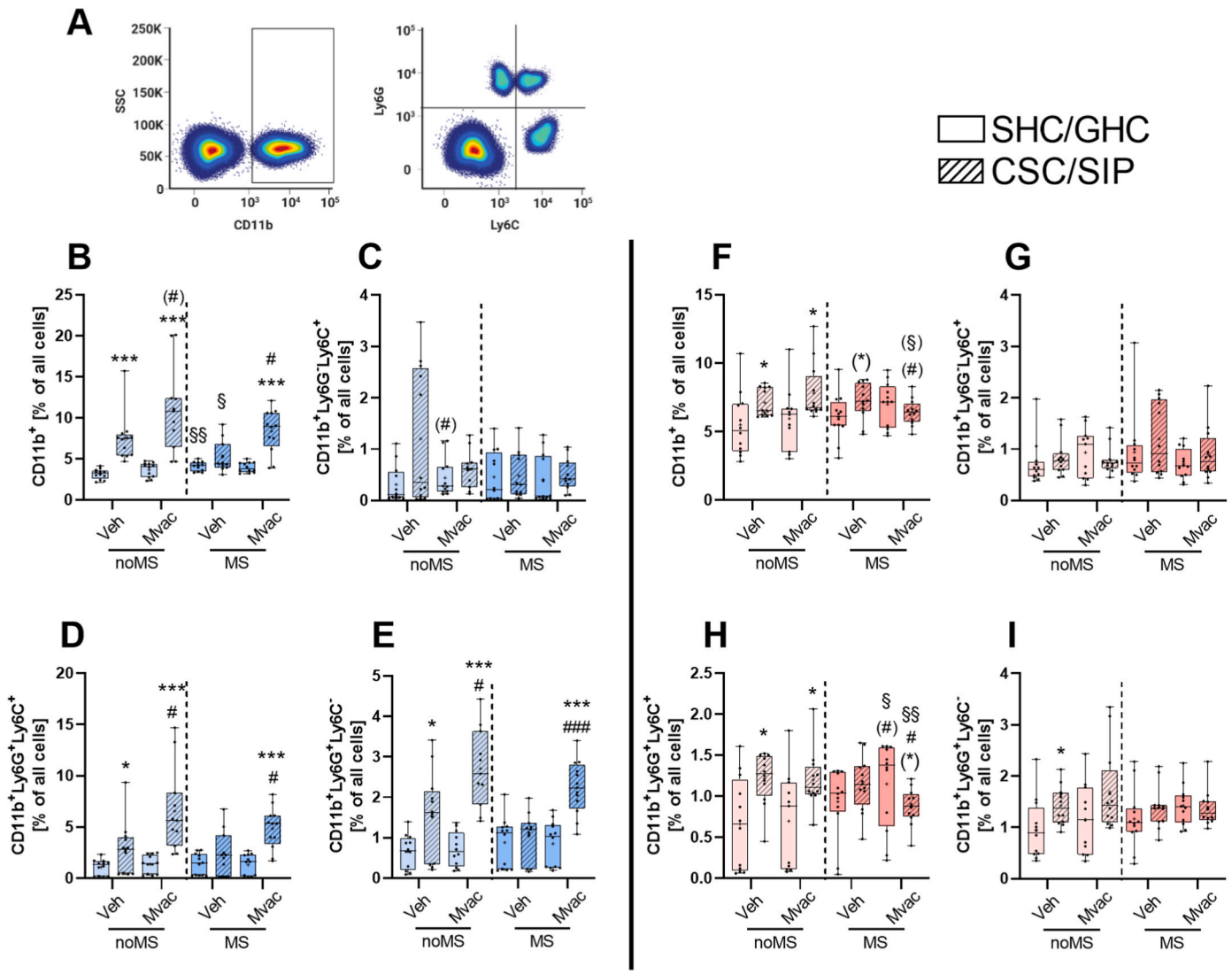

Fig. 5. Effects of ELA, *M. vaccae* and CAS on splenocyte subpopulations. (A) Illustration of the gating scheme for CD11b⁺ subpopulations. (B–E) Flow cytometric analysis of total splenocytes in male mice: overall percentage of (B) myeloid cells (CD11b⁺), (C) monocytes/MO-MDSCs (CD11b⁺Ly6G⁻Ly6C⁺), (D) PMN-MDSC cells (CD11b⁺Ly6G⁺Ly6C⁺) and (E) neutrophils (CD11b⁺Ly6G⁺Ly6C⁻). (F–I) Flow cytometric analysis of total splenocytes in female mice: overall percentage of (B) myeloid cells (CD11b⁺), (C) monocytes/MO-MDSCs (CD11b⁺Ly6G⁻Ly6C⁺), (D) PMN-MDSC cells (CD11b⁺Ly6G⁺Ly6C⁺) and (E) neutrophils (CD11b⁺Ly6G⁺Ly6C⁻). ELA was induced by MS in both sexes; CAS was induced by CSC in male and SIP in female mice. Non-parametric data are presented as box-plots including individual values, with solid line representing the median and plus sign representing the mean for each data-set. Lower boxes indicate 25th, upper boxes 75th percentile; minimum (lower error bar), maximum (upper error bar) as well as possible outliers (closed circles beyond the percentiles) are also shown. (*) $p \leq 0.08$, *$p \leq 0.05$, ***$p \leq 0.001$ versus respective SHC/GHC condition; (#) $p \leq 0.08$, #$p \leq 0.05$, ###$p \leq 0.001$ versus respective Mvac condition; §$p \leq 0.05$, §§$p \leq 0.01$ versus respective noMS condition. For detailed statistical effects see Results section and SupTab.1. Abbreviations: CSC, chronic subordinate colony housing; GHC, group-housed control; MDSCs, myeloid-derived suppressor cells; MO, monocyte-like; MS, maternal separation; Mvac, *M. vaccae*, *Mycobacterium vaccae;* noMS, no maternal separation; PMN, polymorphonuclear; SHC, single-housed control; SIP, social instability paradigm; Veh, vehicle (borate-buffered saline).

2002; Wigger and Neumann, 1999). In contrast, while MS-Veh and noMS-Veh **males** in the present study did not differ in the time spent in the inner zone during OF1 exposure, MS-Veh relative to noMS-Veh mice even spent more time in direct contact with the NO during NO1 testing, together suggesting an anxiolytic rather than anxiogenic effect of MS in male mice. However, as the time spent in direct contact with the empty cage during SPAT1 exposure, which also represents a NO, was comparable between all groups, the anxiolytic MS effect detected during NO1 exposure in male mice seems to be rather weak. One possible mechanism underlying these contrasting effects of MS on general anxiety in male mice might be that mice in the current study, in contrast to the other studies reported above, were repeatedly administered with BBS via the s.c. route, as these mice served as vehicle controls for mice administered repeatedly with *M. vaccae* for promotion of immunoregulation and, thus, stress resilience. According to the match-mismatch hypothesis (Nederhof and Schmidt, 2012), individuals exposed to repeated negative events during early life are able to better cope with stressful events during later life. Thus, MS in combination with repeated s.c. administrations might predispose male mice in the present study to show less anxiety-related behavior during more stressful NO1 exposure, but not less stressful OF1 exposure, relative to respective noMS mice. Noteworthy, a comparable overall distance travelled during OF1 exposure between all groups of male mice indicated that general locomotion at 7 weeks of age was neither affected by MS nor *M. vaccae* administrations, which represents an important prerequisite for locomotion-dependent behavioral tests.

Interestingly, besides a comparable overall distance travelled during OF2 exposure between all groups, indicating that general locomotion

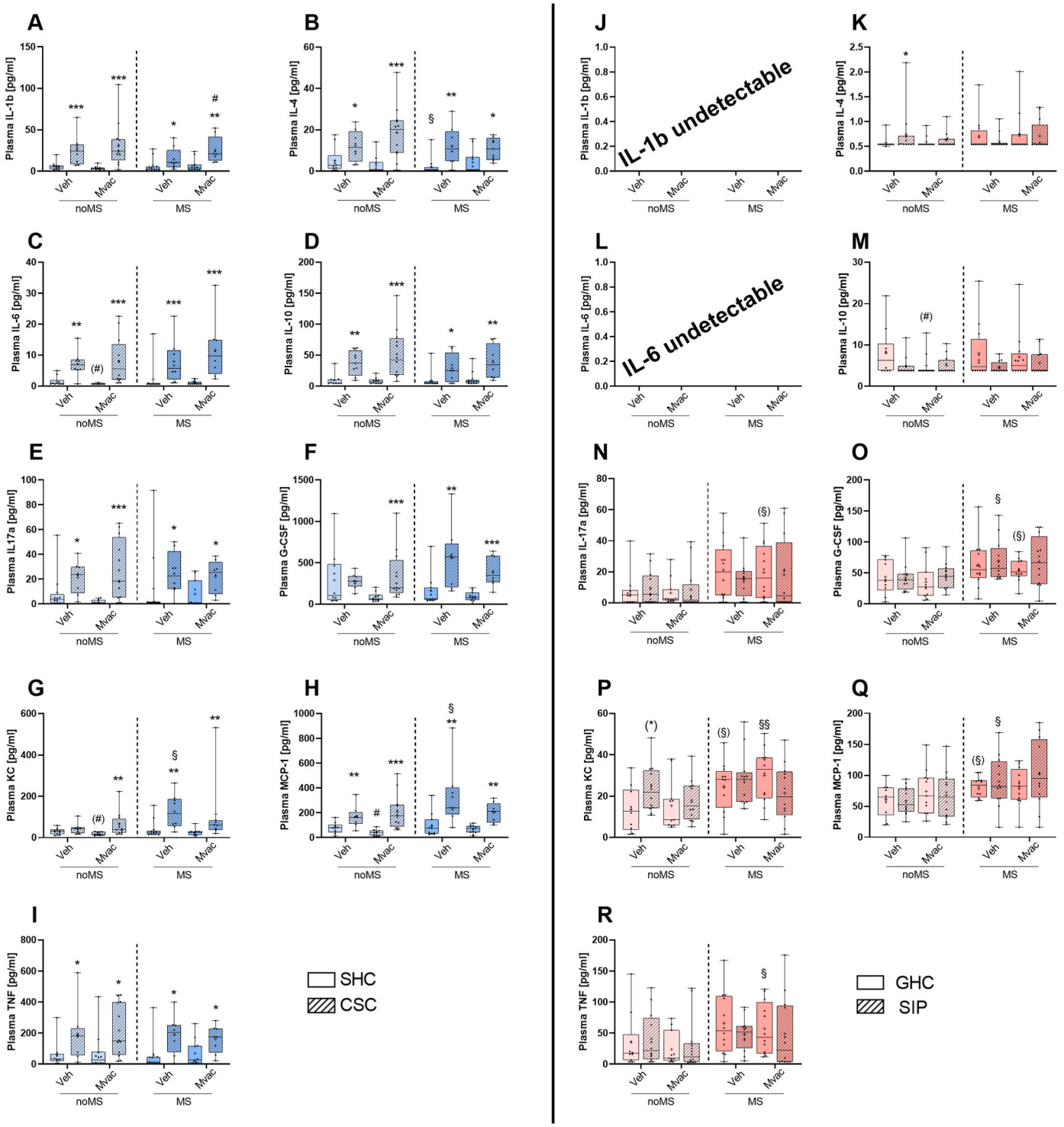

Fig. 6. Effects of ELA, *M. vaccae* and CAS on plasma cytokines. (A–I) Plasma cytokine concentrations in male mice: (A) IL-1β, (B) IL-4, (C) IL-6, (D) IL-10, (E) IL-17a; (F) G-CSF, (G) KC, (H) MCP-1 and (I) TNF. (J–R) Plasma cytokine concentrations in female mice: (J) IL-1β, (K) IL-4, (L) IL-6, (M) IL-10, (N) IL-17a; (O) G-CSF, (P) KC, (Q) MCP-1 and (R) TNF. ELA was induced by MS in both sexes; CAS was induced by CSC in male and SIP in female mice. Non-parametric data are presented as box-plots including individual values, with solid line representing the median and plus sign representing the mean for each data-set. Lower boxes indicate 25th, upper boxes 75th percentile; minimum (lower error bar), maximum (upper error bar) as well as possible outliers (closed circles beyond the percentiles) are also shown. (*) $p \leq 0.08$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus respective SHC/GHC condition; (#) $p \leq 0.08$, #$p \leq 0.05$ versus respective Mvac condition; (§) $p \leq 0.08$, §$p \leq 0.05$, §§$p \leq 0.01$ versus respective noMS condition. For detailed statistical effects see Results section and SupTab.1. Abbreviations: CSC, chronic subordinate colony housing; G-CSF, granulocyte-colony stimulating factor; GHC, group-housed controls; IL, interleukin; KC, keratinocyte chemoattractant; MCP-1, monocyte chemoattractant protein-1; MS, maternal separation; Mvac, *M. vaccae*, *Mycobacterium vaccae;* noMS, no maternal separation; SHC, single-housed control; SIP, social instability paradigm; TNF, tumour necrosis factor; Veh, vehicle (borate-buffered saline).

also at 10 weeks of age was neither affected by MS, *M. vaccae,* nor CSC, no differences were revealed between all groups for the time spent in the inner zone during OF2 and the time in the contact zone during NO2 exposure. The latter suggests that the anxiolytic MS and *M. vaccae* effects observed during OF1/NO1 exposure at the age of 7 weeks were only transient in nature. This hypothesis is also supported by a comparable time in direct contact with the empty cage during SPAT2 exposure between all groups. Together with data from others indicating that MS is not consistently increasing general anxiety-related behavior in adult rodents (Millstein and Holmes, 2007; Romeo et al., 2003; Tractenberg et al., 2016), our data suggest that the time of behavioral testing subsequent to MS exposure might explain why MS just in some studies promotes general anxiety in rodents. Moreover, as MS in males did not cause a behavioral phenotype characterized by increased general anxiety levels, the protective effects of repeated s.c. administrations of *M. vaccae* subsequent to MS exposure on MS-induced general anxiety could not be reliably interpreted in the present study. However, MS-Mvac male mice spent more time in the inner zone during OF1 exposure compared to respective noMS males, indicating at least a mild anxiolytic effect of *M. vaccae* in male MS mice. Noteworthy, although CSC in un-injected mice reliably causes a behavioral phenotype characterized by increased anxiety-related behavior during elevated plus-maze, light-dark box, OF/NO, elevated platform and SPAT exposure (Langgartner et al., 2015, 2017; Reber et al., 2007, 2016a; Slattery et al., 2012; Uschold-Schmidt et al., 2012), CSC in the present study neither promoted general anxiety-related behavior in the OF/NO2 nor the SPAT2. As we have already reported earlier that mice repeatedly administered with Veh do not show a reliable CSC-induced increase in general anxiety (Amoroso et al., 2019), one possible mechanism underlying the lack of the typical anxiogenic CSC effects in MS and noMS mice of the present study might be that mouse handling required for the repeated administrations of Veh is per se stressful for the mice, masking general anxiogenic effects of CSC. Importantly, as CSC in males did not cause a behavioral phenotype characterized by increased general anxiety levels, the protective effects of repeated s.c. administrations of *M. vaccae* prior to CSC exposure on CSC-induced general anxiety could not be reliably interpreted in the present study.

In contrast to the aforementioned mild anxiolytic MS effects, MS-Veh in contrast to noMS-Veh males did not prefer a wire-mesh cage containing a male social stimulus mouse over an empty cage during SPAT1, indicating a MS-induced lack of social preference (Slattery et al., 2012). Of note, both depression and anxiety disorders are often accompanied by social phobia (Schneier et al., 1992), and a decrease in social interest and lack of social preference have been reported to either reflect enhanced depression-related behavior (Berton et al., 2006; Krishnan et al., 2007) or signs of social anxiety (Kalueff et al., 2006). Importantly, this MS-induced lack of social preference was absent in MS males repeatedly administered s.c. with *M. vaccae* subsequent to MS exposure, indicating that *M. vaccae* is able to prevent or reverse development of MS-induced social deficits in male mice. In contrast, repeated s.c. administrations of *M. vaccae* prior to CSC were not preventive against CSC-induced social deficits, indicated by an increased time in the direct contact zone surrounding the social (i.e., male) vs. empty cage during SPAT2 in all SHC but not respective CSC groups, as well as by a decreased time in direct contact with the social cage (i.e., male) in both MS-Mvac-CSC and noMS-Mvac-CSC mice compared with respective SHC mice. Importantly, while the CSC-induced lack of social preference is in line with our own previous studies in untreated (Slattery et al., 2012) as well as repeatedly s.c. (Amoroso et al., 2020; Reber et al., 2016b) and i.g. (Langgartner et al., 2023) administered (i.e., Veh) mice, suggesting that CSC effects on social anxiety are stronger than on general anxiety, the lack of stress-protective effect of *M. vaccae*, at least at the first glance, is in contrast to our own previous data (Amoroso et al., 2020; Reber et al., 2016b). However, a closer look at our earlier studies reveals that the protective effect of s.c. *M. vaccae* were rather mild and hard to interpret, as CSC was not reliably reducing social preference in Veh mice (Amoroso et al., 2020; Reber et al., 2016b). Finally, as MS exposure did not result in social deficits during SPAT2 exposure at the age of 10 weeks, MS-induced and *M. vaccae*-dependent social deficits, detected at 7 weeks of age, seem to be transient only.

Despite the fact that experiments in **female** mice are still rare, increased general anxiety-like behavior has been reported also in females exposed to ELA (Bailoo et al., 2014) or CAS (Bailoo et al., 2014; Schmidt et al., 2010a; Yohn et al., 2019). However, in contrast to these studies, no effects of MS, SIP or the combination of both on general and social anxiety have been found in females during OF/NO1 and OF/NO2, as well as SPAT1 and SPAT2 exposure in the present study, making it further not possible to reliably interpret the protective effects of repeated s.c. administrations of *M. vaccae* on MS and/or SIP-induced general and social anxiety. One possible explanation for the lack of any stress effects on general and social anxiety might be due the stressful handling of the females during substance administrations, as aforementioned in males (Amoroso et al., 2020; Reber et al., 2016b). Of particular note and in line with data from others (Tsuda and Ogawa, 2012), female experimental mice were exposed to a female social stimulus during SPATs, since we could show that female mice display social avoidance towards a male stimulus mouse..

4.2. Effects on the HPA axis

In line with our earlier studies (Veenema et al., 2008), MS exposure of **males** in the present study did not cause adrenal enlargement, suggesting that MS does not result in long-lasting HPA axis activation in male mice. In support of these findings, pituitary weight was also not increased in MS males compared with respective noMS males. In contrast, adrenal weight of male mice exposed to CSC was increased compared with respective SHC mice in all experimental groups, indicating that prior MS exposure did not affect the chronic HPA axis activation typically found in CSC mice and that repeated administrations of *M. vaccae* were not able to prevent CSC-induced adrenal enlargement, as shown previously by our group (Langgartner et al., 2023). Noteworthy, adrenal enlargement has been shown to be the most predictive biomarker for classification and class prediction in the CSC paradigm (Langgartner et al., 2018a) and a typical sign of chronic stress (Langgartner et al., 2015). Interestingly, although prior MS exposure alone did not potentiate CSC effects on adrenal and pituitary weight in Veh-administered males, MS aggravated the CSC-induced increase in adrenal weight and enabled CSC to promote pituitary enlargement in males repeatedly administered with *M. vaccae*. As CSC mice of the MS-Mvac group further showed an increased bite score (for details see below) compared with the respective Veh group, a more severe CSC-induced HPA axis activation in this group might simply reflect a counter-regulatory mechanism to restrain wounding-induced skin inflammation.

In contrast with the aforementioned findings in males, neither MS nor SIP affected pituitary and adrenal weight in **females**. While little is known about long-lasting MS effects on pituitary and adrenal weights in female mice (Weiss et al., 2011), SIP has been shown to promote adrenal enlargement and, thus, chronic HPA axis activation, in female mice (Schmidt et al., 2010a). Importantly, despite the fact that MS and SIP in females did not affect emotionality and HPA axis parameters, given the prominent effects of MS and/or SIP on *in vitro* spleen cell viability and splenocyte composition discussed in detail below, we can exclude that MS and/or SIP did not reliably induce psychosocial stress in females of the current study.

4.3. Effects on the spleen

Spleen weight was not affected by prior MS exposure in **male** mice, which is in line with what has been reported earlier (Savignac et al., 2011). As the percentage of myeloid cells in the spleen was increased in MS vs. noMS males in the Veh-SHC group, these data suggest that

adaptive immune cells are emigrating from the spleen, thereby preventing MS-induced splenomegaly. In support of this hypothesis, our own previous findings indicate that a CSC-induced increase in myeloid splenocytes is also accompanied by a reduction in splenic T and B cells (Foertsch et al., 2017). Importantly, spleen weight was increased in CSC mice of all groups, which is in line with what has been reported in various psychosocial stress models allowing direct physical contact and bite wounding (Avitsur et al., 2001; Foertsch et al., 2017; Foertsch and Reber, 2020; Kempter et al., 2023). In detail, our recent findings support the hypothesis that stress-induced neutrophils, polymorphonuclear (PMN)-myeloid-derived suppressor cells (MDSCs) and monocytes/monocyte-like (MO)-MDSCs get primed and activated locally in the bone marrow, emigrate into the peripheral circulation and subsequently, if CSC is accompanied by significant bite wounding, accumulate in the spleen, promoting splenomegaly (Kempter et al., 2023). Here, PMN-MDSCs and monocytes/MO-MDSCs upregulate TLR4 expression, which exclusively in PMN-MDSCs promotes NF-κB hyperactivation upon LPS-stimulation, thereby exceeding the anti-inflammatory capacities of GCs and resulting in GC resistance (Kempter et al., 2023). In line with these findings, the CSC-induced increase in splenic myeloid cells, neutrophils, and PMN-MDSCs, spleen weight and *in vitro* splenocyte GC resistance were significantly less pronounced or even absent in the MS-Veh group, which was accompanied by a significantly lower bite score in CSC mice of the MS-Veh relative to MS-Mvac mice. Importantly, repeated s.c. administrations of *M. vaccae* prior to CSC in the present study were not protective against the CSC-induced increase in splenic myeloid cells in general and of neutrophils and PMN-MDSCs in particular and, consequently, splenomegaly. This is in line with another recent study from our group, in which repeated i.g. administrations of *M. vaccae* prior to CSC were not protective against CSC-induced splenic invasion of neutrophils, PMN-MDSCs and monocytes/MO-MDSCs, accompanied by splenomegaly (Kempter et al., 2023). However, in contrast to repeated i.g. administrations of *M. vaccae* (Langgartner et al., 2023), repeated s.c. administrations of *M. vaccae* in the present study were not protective against the CSC-induced increase in basal and LPS-induced *in vitro* splenocyte viability in both MS and noMS mice, suggesting that this stress-protective effect of *M. vaccae* is dependent on the application route. Application route-dependent effects of *M. vaccae* have been also reported and discussed earlier by our group (Amoroso et al., 2019, 2020; Kempter et al., 2021). Of note, CSC in the present study did not increase basal and LPS-induced as well as delta (LPS-basal) spleen cell viability in the noMS-Veh group, which is in contrast to our own earlier findings obtained in CSC-exposed mice bought as adolescents from Charles River (Kempter et al., 2021, 2023). These data support the hypothesis that CSC-induced changes in splenocyte *in vitro* cell viability are sensitive to inter- and intra-laboratory environmental variations, especially during early life rearing, as has been reported recently by others (Jaric et al., 2022; Savignac et al., 2011). Importantly, we have shown earlier that wounding during CSC exposure correlates positively with re-active coping (Foertsch et al., 2017). However, as repeated s.c. administrations of *M. vaccae* rather promote pro-active coping in CSC mice (Amoroso et al., 2020; Reber et al., 2016b), it is unlikely that the increased bite score in MS-Mvac-CSC mice seen in the current study is mediated by a shift towards re-active coping behavior in this experimental group. In **female** mice, MS decreased spleen weight in the Veh-GHC group of the present study, which is in contrast to the study in males referenced above revealing no MS effects on spleen weight (Savignac et al., 2011). As the percentage of myeloid cells in the spleen was comparable between MS and noMS females in the Veh-SHC group, these data again suggest that adaptive immune cells, as discussed above in males, are emigrating from the spleen, thereby promoting a reduction in spleen weight. In line with the hypothesis that MS promotes splenic emigration of adaptive immune cells, MS in Mvac-GHC mice resulted in an increased percentage of PMN-MDSCs ($CD11b^{+}Ly6G^{+}Ly6C^{+}$) compared with respective noMS females despite an unaffected spleen weight. As the percentage of PMN-MDSCs in MS-Mvac-GHC mice was significantly lower compared with respective Veh mice, repeated s.c. administrations of *M. vaccae* seem to be protective against MS-induced splenic invasion of PMN-MDSCs in females. However, further studies are required to investigate this phenomenon in more detail. In line with our findings in males, CAS induced by SIP also in females caused an increased percentage of splenic myeloid cells and PMN-MDSCs in both the noMS-Veh and noMS-Mvac groups. However, as this increase was not accompanied by a SIP-induced increase in spleen weight, again emigration of adaptive immune cells is likely. Of note, the absence of the SIP-induced splenomegaly is not in contrast to findings showing an increased spleen weight in female mice exposed to social defeat stress, which as in males allows direct physical interaction and physical contact (Yin et al., 2019). Importantly, in contrast to total myeloid cells and PMN-MDSCs and in contrast to our findings in males exposed to CSC, the SIP-induced increase in the percentage of neutrophils was only detectable in the noMS-Veh group, but absent in the noMS-Mvac group, suggesting that repeated s.c. administrations of *M. vaccae* in females are able to prevent SIP-induced splenic invasion of neutrophils. Interestingly, and this is again in contrast to our findings in males exposed to CSC, this neutrophil splenic invasion is not accompanied by an increased splenocyte *in vitro* viability, suggesting that these neutrophils in contrast to CSC males seem not to be primed and activated in the BM. Moreover, the SIP-induced increase in total myeloid cells, PMN-MDSCs and neutrophils in the noMS-Veh group, which has been also reported in female mice exposed to social defeat (Yin et al., 2019), is absent in the MS-Veh mice, suggesting prior MS exposure to be preventive against SIP-induced splenic invasion of various myeloid subpopulations, while *M. vaccae* is only protective against SIP-induced splenic invasion of neutrophils. As bite wounds were undetectable in any of the female experimental groups, wounding dependent mechanisms are unlikely to underlie the MS and/or SIP-induced splenic immigration of different myeloid subsets. The fact that β-adrenergic agonists have been shown to increase BM myelopoiesis, myeloid BM emigration and subsequent splenic immigration of these cells causing splenomegaly (McKim et al., 2018) supports sympathetic nervous system-dependent mechanisms instead of wounding-dependent ones to mediate stress-induced splenomegaly in females. Of note, splenic *in vitro* GC sensitivity was neither affected by MS, SIP nor by repeated *M. vaccae* administrations in female mice.

4.4. Effects on plasma cytokines

In the current study, MS did not severely affect plasma cytokine levels in **male** mice of the Veh-SHC group, except a reduction of plasma IL-4 compared with respective noMS mice. As this MS-induced IL-4 reduction was absent in the respective Mvac group, repeated s.c. administrations of *M. vaccae* seem to be protective against ELA-induced changes in plasma cytokines. The latter is in contrast to what we know from a recent study from our group, indicating that repeated i.g. administrations of *M. vaccae* did not protect against the CAS-induced elevations in plasma cytokines. However, whether this discrepancy is due to ELA vs. CAS or due to s.c. vs. i.g. administration issues needs to be assessed in future studies. In support of the former and in line with our own previous studies (Haffner-Luntzer et al., 2019; Langgartner et al., 2018b, 2023), CSC in the current and previous studies reliably increased various proinflammatory cytokines and this was not prevented by repeated s.c. *M. vaccae* administrations. Of note, increased IL-6 plasma levels have been also reported following social defeat stress (Avitsur et al., 2002), while inescapable foot shocks even increased IL-1β, IL-6, TNF, IL-10, IL-17a and G-CSF plasma levels (Cheng et al., 2015). Interestingly, the CSC-induced increase in plasma KC and MCP-1 concentrations was more pronounced in MS-Veh relative to noMS-Veh males, with s.c. *M. vaccae* protecting against the additive effects of MS and CSC, but not against CSC effects alone. Of note, CSC did neither increase anti-inflammatory IL-10 plasma concentrations in any group, nor G-CSF in the noMS-Veh group, although the latter has been shown

before (Langgartner et al., 2018b).

In **female** mice of the current study, MS increased plasma MCP-1 concentration in the Veh-GHC but not the Mvac-GHC group, suggesting that repeated s.c. administrations of *M. vaccae* are protective against MS-induced systemic increase in MCP-1 levels. In contrast to this hypothesis, repeated s.c. administrations of *M. vaccae* were not protective against MS-induced systemic KC levels, indicated by an MS-induced increased in plasma KC concentration in both the Veh-GHC and the Mvac-GHC group. A MS-induced increase of IL-17a, G-CSF and TNF in specifically the Mvac-GHC group suggests that s.c. *M. vaccae* even facilitates the MS-induced increase of certain proinflammatory cytokines. In contrast to the pronounced and reliable CSC-induced increase in various proinflammatory cytokines in males reported in the present and earlier studies (Haffner-Luntzer et al., 2019; Langgartner et al., 2018b, 2023), SIP in female mice only increased IL-4 plasma cytokine levels in the noMS-Veh group but not the respective Mvac group, indicating protective effects of repeated s.c. administrations of *M. vaccae* on SIP-induced systemic increase in IL-4. Interestingly, G-CSF plasma levels were increased by MS in the Veh-SIP but not Mvac-SIP group indicating protective effects of s.c. *M. vaccae* against these additive effects of MS and SIP. Of note, repeated s.c. *M. vaccae* decreased plasma IL-10 levels in noMS-GHC females compared with respective Veh mice. Moreover, despite the fact that increased plasma IL-6 levels have been reported in female mice exposed to social defeat stress (Yin et al., 2019), plasma IL-1β and IL-6 concentrations were below the respective detections limits of the Bio-Plex Pro cytokine assay.

4.5. Limitations and conclusions

One limitation of the current study is that CAS in adult male and female mice was induced by different stress paradigms, making it difficult to draw any reliable conclusions with respect to sex-specific differences in the vulnerability to CAS as well as to the combination of ELA and CAS. In contrast, as both male and female offspring have been exposed to identical MS protocols, conclusions drawn with respect to sex-specific differences in the vulnerability to ELA are reliable and, therefore, justified. As development of psychosomatic disorders in humans is often associated with chronic stressors, which are mostly psychosocial in nature, it was our aim in the present study to induce ELA but also CAS in both sexes by psychosocial stress paradigms. While for the induction of ELA in both sexes the MS paradigm was adequate, respective internationally-accepted CAS models for male mice are predominantly based on territorial aggression, social defeat and establishment of social hierarchies (Foertsch and Reber, 2020; Langgartner et al., 2015; Reber et al., 2016a), while respective paradigms for females are based on social instability (Yohn et al., 2019) and social isolation (Madden et al., 2013; Palanza et al., 2001). This holds true also for the two murine CAS models chosen in the present study, the CSC paradigm in males (Foertsch and Reber, 2020; Langgartner et al., 2015; Reber, 2012; Reber et al., 2016a) and the SIP model in females (Schmidt et al., 2008; Sterlemann et al., 2008; Yohn et al., 2019). As chronic stress effects are further known to be age-dependent (Kempter et al., 2021; Laviola et al., 2002; Lotan et al., 2018; Pardon et al., 2000), it is noteworthy in this context that the here used paradigms do not just differ in the type of psychosocial stressor but also in the duration of the paradigms, with the CSC model lasting 19 days and the SIP model 7 weeks. Non-social stressors, as for instance immobilization, acoustic stress or electric shocks, would allow exposure of both sexes to the identical stress paradigm, but are unnatural for both mice and humans and, thus, of less scientific and translational value.

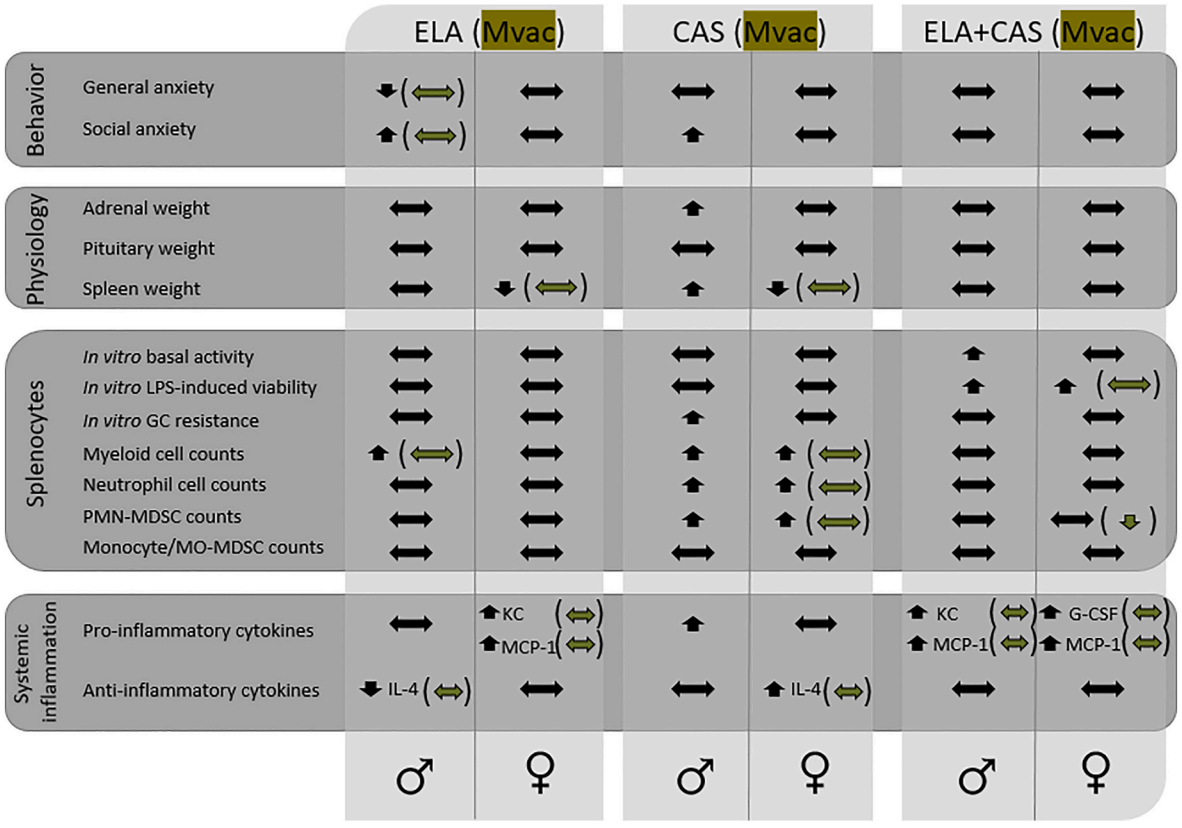

Fig. 7. Summary of findings. ELA was induced by MS in both sexes; CAS was induced by CSC in male and SIP in female mice. Abbreviations: CAS, chronic adult stress; ELA, early life adversity; GC, glucocorticoid; G-CSF, granulocyte colony-stimulating factor; IL-4, interleukin-4; KC, keratinocyte chemoattractant; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MO-MDSC, monocyte/monocyte-like myeloid-derived suppressor cell; Mvac, *Mycobacterium vaccae*; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell.

Taken together (Fig. 7), our data indicate that the effects of MS alone were surprisingly mild in both sexes, with by trend increased systemic KC and MCP-1 levels despite reduced spleen weight in females and transient social deficits, increased splenic myeloid counts and decreased plasma IL-4 levels in males. Importantly, our data further indicated that all these mild and sex-specific MS effects, except the female-specific increase in systemic MCP-1 levels, were prevented/reversed in male and female MS mice by repeated s.c. administrations with *M. vaccae* subsequent to MS exposure. Although a direct comparison of CSC effects in males and SIP effects in females, as detailed above, is not possible, the presence of social deficits, chronic HPA axis activation, splenomegaly accompanied by increased *in vitro* splenocyte reactivity, GC resistance, increased counts of splenic total myeloid cells, PMN-MDSCs and neutrophils together with increased plasma levels of various pro- but not anti-inflammatory cytokines in CSC-exposed male mice, as well as increased splenic total myeloid cells, PMN-MDSCs and neutrophils together with increased plasma IL-4 levels in SIP-exposed female mice verified that both CAS paradigms reliably worked in the present study.

In contrast to the pronounced and sex-independent protective effects of repeated s.c. *M. vaccae* administrations against the negative outcomes of ELA, *M. vaccae* administered subsequent to ELA but prior to CAS did only protect against the SIP-induced increase in splenic neutrophil counts and in plasma IL-4 levels in females, while all other negative stress effects of CAS in both sexes were not affected.

Finally, repeated s.c. administrations of *M. vaccae* were protective against the mild additive effects of ELA and CAS on plasma KC and MCP-1 or G-CSF and MCP-1 levels in males and females, respectively, as well as on splenic LPS-induced *in vitro* cell viability in females. Prior MS exposure further protected females against SIP-induced increases in splenic total myeloid cells, PMN-MDSCs and neutrophils, with SIP in *M. vaccae*-administered MS mice even resulting in a reduction in the percentage of splenic PMN-MDSCs.

CRediT authorship contribution statement

Giulia Mazzari: performed the experiments, did the statistical analysis and created the figures, wrote the manuscript. **Christopher A. Lowry:** wrote the manuscript. **Dominik Langgartner:** planned the study, performed the experiments, wrote the manuscript. **Stefan O. Reber:** planned the study, wrote the manuscript.

Declaration of competing interest

GM, DL and SOR have nothing to declare. CAL is Cofounder, Board Member, and Chief Scientific Officer of Mycobacteria Therapeutics Corporation, and is a member of the faculty of the Integrative Psychiatry Institute, Boulder, Colorado, the Institute for Brain Potential, Los Banos, California, and Intelligent Health Ltd, Reading, UK..

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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