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Investigating the stress-protective effects of *Mycobacterium vaccae* administration in a mouse model of PTSD

Studio degli effetti antistress della somministrazione di *Mycobacterium vaccae* in un modello murino di PTSD

Relatori:
Chiar.mo Prof. STEFAN OSCAR REBER
Chiar.mo Prof. ANDREA SGOIFO

Controrelatore:
Chiar.mo Prof. LUCA CARNEVALI

Laureanda:
GIULIA MAZZARI

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Abstract

In modern society, chronic psychosocial stress represents a risk factor for many somatic, as well as mental disorders, such as inflammatory bowel disease and posttraumatic stress disorder (PTSD). Of note, these stress-related disorders share inflammation as a common background. Stress is also known to activate the immune system and to promote chronic low-grade inflammation. Thus, immunoregulatory-based approaches are hypothesized to be protective against stress-associated pathologies. According to the "Old Friends hypothesis", the increased incidence in inflammatory disorders in modern day society may stem from a reduced contact with microorganisms with immunoregulatory – and therefore – anti-inflammatory properties with which humans came into daily contact during evolutionary times. One such microorganisms is *Mycobacterium vaccae* (*M. vaccae*), an environmental saprophyte with immunoregulatory and stress protective properties.

Previous studies have shown that *M. vaccae* administration has positive effects on stress-induced inflammation and general anxiety in the chronic subordinate colony housing (CSC) paradigm, a mouse model of PTSD, with the effect size being slightly dependent on the route of administration (subcutaneous, intranasal) and on the time of administration (prior to or during chronic psychosocial stress exposure). Therefore, the aim of this thesis was to assess whether the stress protective effects of intragastric (i.g.) administered *M. vaccae* can be potentiated given prior to or during chronic psychosocial stress exposure.

In the present thesis it was shown that i.g. M. vaccae determines mild stress-protective effects on mice: differences were observed between groups, depending on whether the bacterium was administered prior to- or during- CSC exposure. However, overall the findings of this work broaden the general knowledge on using bioimmunoregulatory approaches to promote stress resilience.

CHAPTER

Introduction 1

1.1 Stress physiology

The stress response is regulated by two major systems: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenomedullary system. The first one stimulates the adrenal cortex to release glucocorticoids (GC) – such as cortisol in humans and corticosterone in rodents – into the blood. The second system influences the hormonal stress-response through the adrenal medulla, a part of the sympathetic nervous system (SNS) that secretes mainly adrenaline.

The amygdala is one of the first brain areas to process perceived stressful events. This part of the temporal lobe then influences the activity of other key brain areas in the stress response, like the hypothalamus, which controls vegetative functions by influencing the autonomic nervous system (ANS). The latter has three components: the sympathetic, the parasympathetic and the enteric nervous system. The first one stimulates the so-called *fight or flight* response (Cannon, 1915) and increases the state of arousal in order to sustain moments of increased energetic demands.

The second one stimulates the so-called *rest and digest* response and antagonizes the activity of the sympathetic nervous system. Lastly, the third division of the ANS is the enteric nervous system, which has connections with the central nervous system (CNS). It contains neuronal circuits that detect and control the physiological conditions of the gastrointestinal tract.

After the amygdala sends a distress signal, the hypothalamus activates the sympathetic nervous system, sending signals to the adrenal medulla. The latter responds by releasing adrenaline and noradrenaline into the bloodstream: this results in the reduction of gastrointestinal as well as reproductive organ activity (Hardy & Pollard, 2006) and in an increased heart rate, blood pressure and breathing rate. Meanwhile, glucose and triglycerides are released from storage sites in the body, allowing mobilization of energy (Silverman et al., 2005).

Such hormone interactions have evolved to adjust internal environment to maintain an equilibrium: the so-called homeostasis (Silverman et al., 2005). However, stress can disrupt this internal stability. Moreover, together with homeostasis, allostasis was introduced by P. Sterling and J. Eyer in 1988: this term indicates the ability of achieving stability through change (McEwen & Wingfield, 2010) and it refers to the process that maintains homeostasis when this one is threatened by stressors. The SNS and HPA axis are two primary allostatic systems: the body turns on an allostatic response for coping with stressors and adaptation, and, when the challenge has passed, it turns off the response. Chronic exposure to elevated levels of GCs results in somatic and psychological pathologies. If allostatic response is limited to the period of stress exposure, it has a protective effect on the body. When the response goes over weeks, months or years, it becomes an allostatic overload which can result in pathophysiological consequences (McEwen & Wingfield, 2010).

1.1.1 Acute and chronic stress

The stress response can be acute or chronic in nature. The classification is based on the duration of the stressor exposure. While acute stress only lasts for minutes to hours, chronic stress is defined to persist from days up to months (Dhabhar, 2006). Stress intensity can be determined by measuring GC or catecholamine levels. GC levels reach their peak plasma concentrations within 15-30 minutes after acute stressor exposure and then decline back to baseline within the next few hours (de Kloet et al., 2005). In contrast, if stressor exposure is prolonged or repeated, it might result in a persistent elevation in GC levels and might have adverse effects on mental and physical health.

1.1.2 Hypothalamic-Pituitary-Adrenal Axis

The HPA axis involves complex interactions between the hypothalamus, the pituitary gland, and the adrenal glands. The HPA axis is involved in functions such as body temperature, digestion, immune system, mood, sexuality, and energy usage, in addition to its role as a component of the fight-or-flight response and the release of hormones such as GCs. Stressor exposure results in the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus. After CRH is secreted into the portal hypothalamic-hypophyseal neurosecretory system and reaches the anterior pituitary gland, adrenocorticotropic hormone (ACTH) is secreted into the blood stream. ACTH binds to melanocortin (MC) 2 receptors on the adrenal cortex and stimulates the zona fasciculata of the adrenal glands to release GCs. GCs also exert a negative feedback loop on the release of CRH from the hypothalamus and on the release of ACTH from the pituitary gland, as well as acting on higher levels in the brain like hippocampus, thereby preventing uncontrolled elevations of CORT levels.

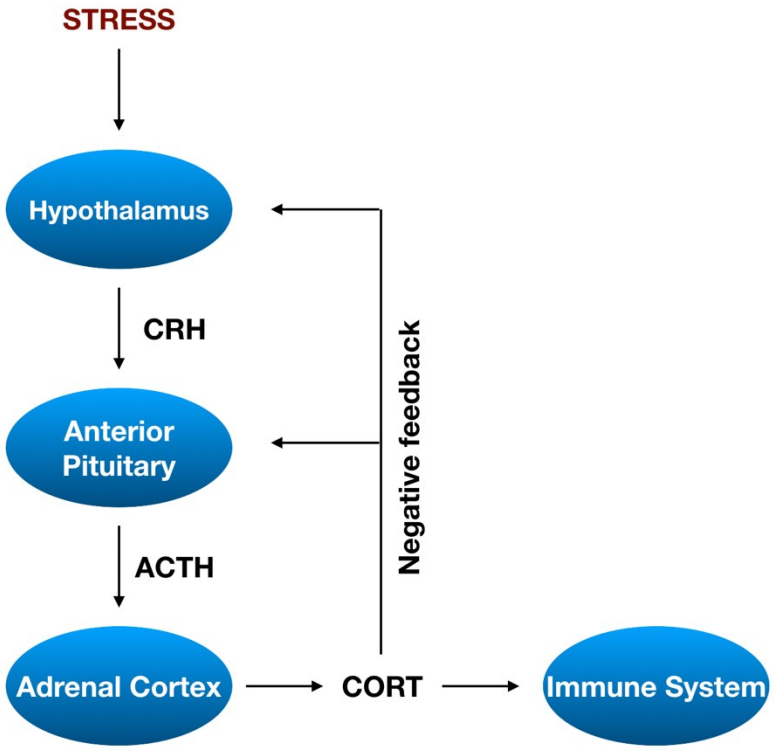

Figure 1 Hypothalamus-pituitary-adrenal (HPA) axis: psychosocial stress triggers the release of corticotropin-releasing hormone (CRH) from the hypothalamus, which stimulates the pituitary to produce adrenocorticotropic hormone (ACTH). ACTH stimulates the secretion of cortisol in humans and corticosterone (CORT) in mice. CORT has negative feedback action on both the hypothalamic release of CRH and the pituitary release of ACTH.

1.2 Immune system

The immune system plays a critical role in stress-associated disorders. Defense against microbes and pathogens is mediated by the early reactions of innate immunity and the later responses of adaptive immunity: the innate immune system gives a general primary defense, while the adaptive immune system is antigen specific. The combined effects of these two arms of the immune system ensures an effective defense against pathogens.

1.2.1 Innate immune system

The innate immune system is not specialized for any particular pathogen, but rather responds to molecules that do not belong to the body, which are known as non-self. It can respond against virtually any kind of non-self molecules, whether or not those were previously encountered.

This system consists of skin and mucous barriers, leukocytes (macrophages, neutrophils, basophils, eosinophils, mast cells, natural killer cells) and proteins (C reactive protein, complement system), and the immune response is activated immediately upon pathogen encounter: this leads to an inflammatory response that increases blood flow to the affected area and guarantees invasion of leukocytes.

These general immune responses can differentiate between self and foreign but do not differentiate between specific types of pathogens (Delves, 2000).

In addition, the complement system is made up of proteins which assist the function of both innate and adaptive immune system by marking and binding pathogens for easier access to phagocytosis and cell lysis. These proteins can have an important function, in chemotactically guiding immune cells to areas where they are needed.

1.2.2 Adaptive immune system

The adaptive immune system is slower in its response to pathogens but shows immunological memory and acts in a targeted manner toward specific antigens. When the antigen is already known, the defense response is more efficient and faster than the innate defense: this is due to the ability of the adaptive immune system to produce memory cells and antigen-specific antibodies.

The two general types of cells in the adaptive immune system are B cells and T cells, both of which derive from hematopoietic precursors found in the bone marrow. B and T cells are

lymphocytes as they reside in their resting state in several lymphoid tissues like lymph nodes and spleen (Delves, 2000).

T cells mature in the thymus and differentiate into several subtypes. During an immune reaction, T lymphocytes develop into specialized cells which include: T helper cells, T killer cells, memory T cells and regulatory T cells.

Helper T cells (T*h* cells) and cytotoxic T cells (T*c* cells) are effector T cells. Clusters of differentiation (CD) allow to differentiate these cells: Th cells express CD4 and Tc cells express CD8 (Hoffmann & Akira, 2013). Tc cells have the major antiviral activity, with the ability to recognize viral molecules on the surface of infected host cells. Moreover, $CD4^+$ Th cells secrete specific cytokines upon activation, which makes possible to classify these cells into different subgroups: Th1 cells initiate the inflammatory response through the secretion of cytokines like IL-2 and IFN-gamma; Th2 cells promote activity of B cells through the secretion of IL-4, IL-5, IL-6 and IL-10; Th17 cells are proinflammatory and produce IL-17 (Delves, 2000).

Regulatory T cells (T_{reg} cells) are $CD4^+$ helper T cells which additionally express CD25 and the transcription factor forkhead box P3 (Foxp3). The differentiation of naïve T cells into T_{reg} cells is dependent on a specific subpopulation of activated dendritic cells known as regulatory dendritic cells (DC_{reg}). T_{reg} cells mainly produce IL-10 and TGF-β, both of which are anti-inflammatory cytokines. Overall, T_{reg} cells suppress inflammatory responses and prevent autoimmune diseases and allergies (Fontenot et al., 2005).

B cells mature in the bone marrow and are then released into the blood. B cells are important for the adaptive defense mechanism, because their activation leads to the production of antibodies specific for a certain antigen (Delves, 2000; Parkin & Cohen, 2001).

1.3 Posttraumatic stress disorder (PTSD)

PTSD is a psychiatric disorder that can occur in people who have experienced or witnessed a traumatic event such as a natural disaster, a serious accident, a terrorist attack, war/combat, rape or other violent personal assault (Brunello et al., 2001; Zohar et al., 2008). Despite the gravity, half of the people who experience a traumatic event and develop PTSD will recover without treatment (Brunello et al., 2001).

PTSD patients keep producing intense, disturbing thoughts and feelings related to their experience that last long after the traumatic event has ended. They may re-experience the event through flashbacks or nightmares; they may feel sadness, fear or anger and they may feel detached or estranged from other people. People with PTSD may avoid situations or people that remind them of the traumatic event, and they may present hyperarousal with consequent impulsiveness, such as negative reactions to something as ordinary as a loud noise or an accidental touch, impaired concentration and sleep alterations (Brunello et al., 2001; Zohar et al., 2008).

Moreover, PTSD comorbidity with other disorders has been observed: overall, major depressive disorder is the most prevalent, then personality disorders in general and finally alcohol and/or drug dependence and abuse (Brady et al., 2000).

Diagnostic criteria for PTSD are defined in the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V), where four symptoms categories have been defined: “intrusive symptoms”, like flashbacks; “persistent avoidance”, referred to thoughts about the trauma; “alteration in cognitions”; “alteration in arousal and reactivity”, like hypervigilance (American Psychiatric Association, 2013).

In summary, for a correct diagnosis of PTSD, the symptoms must be experienced for at least one month and they must impair social and occupational function, as well as other aspects of everyday life (American Psychiatric Association, 2013).

1.3.1 Pathophysiology

PTSD is comorbid with several somatic and affective disorders, of which cardiovascular (Delves, 2000) and autoimmune disorders, like inflammatory bowel disease (IBD) and rheumatoid arthritis (O'Donovan et al., 2015), are the most common.

As previously described, PTSD patients are unable to inhibit aversive memories, since memory and cognition are affected by this pathology. Furthermore, emotional symptoms associated with PTSD can be due to both over-activation or excessive inhibition of limbic structures (Yehuda et al., 2015), but also the over-activation of the SNS during trauma can increase anxiety and arousal in patients (Bremner et al., 2007).

PTSD-affected people also exhibit an impaired GC signaling, showing decreased levels of basal cortisol in plasma, salivary and urinary samples (Miller, 2003). In general, a higher response to stressful situations is shown by patients diagnosed with PTSD due to HPA axis sensitization (Bremner et al., 2007; Brunello et al., 2001; Miller, 2003).

Moreover, higher basal levels of catecholamines suggest a higher SNS activation in PTSD patients. This is supported by an increased heart rate and blood pressure at baseline level and in response to a stressor (Bremner et al., 2007) in PTSD patients.

In addition, PTSD patients often show chronic low-grade inflammation, as they exhibit slightly increased blood levels of pro-inflammatory cytokines, such as IL-1β, IFN-γ and TNF-α (Gill et al., 2008; Spivak et al., 1997; von Känel et al., 2007), and decreased blood levels of anti-inflammatory cytokines like IL-4 (von Känel et al., 2007). Moreover, PTSD patients display a

reduction in the percentage of regulatory T cells (Sommershof et al., 2009), and an increase in the numbers of T helper and cytotoxic T cells (Vidović et al., 2007). Consistent with this chronic low-grade inflammatory status, PTSD patients also show elevated levels of c-reactive protein (CRP) (Heath et al., 2013; Michopoulos et al., 2015; Spitzer et al., 2010), and this also predicts the risk to develop PTSD (Eraly et al., 2014).

1.3.2 Treatments

For some people, the symptoms of PTSD disappear or subside over time. In case this does not happen spontaneously, a professional treatment is needed, in order to recover from psychological distress: both psychotherapeutic approaches and pharmacological medication represent effective evidence-based therapies for PTSD (Yehuda et al., 2015). However, the therapies are limited and mostly focused on the symptoms more than the underlying causes.

Since increasing inflammation is hypothesized to be involved in the pathophysiology of PTSD, showing chronic low-grade inflammatory status with higher levels of pro-inflammatory and lower levels of anti-inflammatory cytokines, counterbalancing the activity of an over-reactive immune system could be a novel approach to treat PTSD symptomatology (Rohleder, 2014).

1.3.3 Chronic subordinate colony housing paradigm

In order to understand the mechanisms underlying stress-related diseases, animal models that reliably mimic psychiatric disorders like PTSD are needed.

The chronic subordinate colony (CSC) housing paradigm has proven to be an adequate mouse model for PTSD (Reber et al., 2016). Similarly to the traumatizing events that promote the development of PTSD in humans, experimental mice are exposed to repeated psychosocial traumatization (i.e. repeated social defeat) during the CSC paradigm. In detail, four

experimental mice are exposed to and cohoused with an aggressive conspecific in its home cage for 20 consecutive days. In order to avoid habituation, the aggressor mouse is changed weekly. Several physiological, behavioral and immunological alterations developed by CSC mice are comparable to the ones described as diagnostic criteria for PTSD (Reber et al., 2016).

From a physiological point of view, CSC mice show an altered HPA axis functionality, expressed as impaired GC signaling with reduced plasma evening but not morning CORT levels compared to single housed control (SHC) mice (Reber et al., 2007).

On the other hand, when they are subjected to acute stress, the HPA axis in CSC mice produces more CORT compared to SHC mice (Uschold-Schmidt et al., 2012). In addition, these changes in HPA axis activity and GC signaling coincide respectively with an increase in weight of pituitary and adrenal glands (Füchsl et al., 2013; Langgartner et al., 2015). Moreover, CSC results in GC resistance of splenocytes *in vitro*, characterized by splenomegaly (Foertsch et al., 2017; Füchsl et al., 2014; Reber et al., 2007).

Another significant comparison between PTSD patients and CSC mice concerns the immune system. In both cases, pro-inflammatory plasma cytokines (IL-6, IFN-γ, TNF-α) are increased (Guo et al., 2012; Maes et al., 1999; Reber et al., 2016). Moreover, the number of T_{reg} cells in blood and in peripheral lymph nodes is decreased (Reber et al., 2016).

CSC paradigm also induces behavioral alterations: PTSD patients show anxiety disorders as well as anxiety-related behaviors are observed in mice exposed to CSC. The anxiogenic effect of CSC results in social anxiety behaviors, reflected by the lack of preference toward unfamiliar conspecifics (Foertsch et al., 2017; Reber et al., 2007; Reber et al., 2016). Furthermore, a decreased locomotor activity during behavioral tests directly following CSC exposure and hyperarousal one week after cessation of CSC exposure can be observed in CSC animals (Langgartner et al., 2015; Slattery et al., 2012).

1.4 Old Friends

The increased incidence of inflammatory conditions like allergies, autoimmunity and chronic inflammatory disorders in human beings during the last century can be explained by the modern living conditions. The "Old friends hypothesis" has been developed by Graham Rook (Rook, 2009), starting from the "Hygiene hypothesis", which explained the increase of allergic diseases with the concomitant improvement of hygiene in modern society (Strachan, 1989). Rook expanded this view, attributing the increased incidence of inflammatory conditions to a lack of exposure to organisms called "old friends" (Rook, 2009; Rook et al., 2013; Rook & Lowry, 2008). These organisms co-evolved with humans (Rook et al., 2004), but nowadays are less common in urban areas compared to rural ones. Most of the old friends are harmless and, therefore, tolerated by the immune system. At the same time, other old friends organisms are not harmless but tolerated by the immune system, because an immune response against them could result in severe tissue damage (Babu et al., 2006; Rook & Lowry, 2008).

A possible way of action of the "old friends" to induce immunoregulation was described by Rook (Rook et al., 2013). Dendritic cells (DC) mature into regulatory DC with the ability of facilitating T_{reg} generation. Afterwards, the increasing number of T_{reg} induced by "old friends" leads to two different immunoregulatory mechanisms mediated partly by the secretion of IL-10 and transforming growth factor (TGF)-β. First, continuing exposure to "old friends" will cause continuous background activation of T_{reg} specific for the "old friends" themselves, resulting in a constant background of bystander suppression. Secondly, DCs also sample self, gut contents and allergens which downregulate the immune system for what concern autoimmunity or allergies (Rook, 2009).

Moreover, it has been observed that chronically increased levels of the pro-inflammatory markers CRP and IL-6 are prevalent in subjects living in developed countries (Gimeno et al., 2009). Interestingly, living in a urban area has shown a higher prevalence for developing

psychiatric disorders when compared to population of rural areas (Peen et al., 2010; Pedersen & Mortensen, 2001). In addition, a correlation between depressed individuals and raised levels of pro-inflammatory cytokines, together with a deficit in T_{reg} cells, was observed (Raison et al., 2010).

As inflammation plays an important role in the negative effects of psychosocial stress, and as chronic psychosocial stress can increase inflammation (Rohleder, 2014), approaches aimed at reducing inflammation may be protective in this context.

Mycobacterium vaccae (*M. vaccae*) is one of the microorganisms called "old friends". It is an abundant soil saprophyte with immunoregulatory properties. A heat-killed preparation of this bacterium induced an increased T_{reg} number, anti-inflammatory cytokine levels and alleviated the symptoms of asthma (Zuany-Amorim et al., 2002). In a recent study it was also shown that CSC-induced aggravation of DSS-induced colitis could be prevented by intranasal administration of *M. vaccae* (Amoroso et al., 2019). Furthermore, antidepressant effects of *M. vaccae* on anxiety-related behaviors in mice were shown (Lowry et al., 2007), and repeated subcutaneous (s.c.) administrations decreased anxiety-related behaviors in CSC mice (Reber et al., 2016).

1.5 Aim of the work

As outlined in detail above, the immune system plays a key role in developing chronic stress-related disorders, including PTSD, and *M. vaccae* has been shown to have pronounced stress-protective effects, at least when given s.c. (Amoroso et al., 2019a, Reber et al., 2016) and i.n (Amoroso et al., 2019b).

However, unpublished studies by the Reber group investigating the effects of intragastric (i.g.) administration of *M. vaccae* either prior to or during CSC only showed mild stress-protective effects.

Therefore, the aim of this thesis was to investigate whether a prolonged repeated intragastric administration of *M. vaccae* both prior to and during chronic psychosocial stress increases its protective effects against negative physiological, immunological and behavioral stress consequences in male mice.

To induce chronic psychosocial stress, the CSC paradigm, a validated mouse model for PTSD, was applied for 21 consecutive days. Overall, a total of four experimental groups are used: *M. vaccae* prior to CSC; *M. vaccae* during CSC; *M. vaccae* prior to and during CSC; Vehicle (BBS) prior to and during CSC.

CHAPTER

Materials and Methods 2

2.1 Ethics statements

The project and all experimental protocols were approved by the Committee on Animal Health and Care of the local government and conducted according to national and international guidelines on the ethical use of animals. All the 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 (C. Kilkenny, 2010).

2.2 Animals

Male specific pathogen free (SPF) C57BL/6N mice, weighing 17-19 g, were used as experimental mice. Male SPF CD-1 mice (weight: 30-35 g), were used as dominant aggressor mice for the CSC paradigm. All mice were purchased from Charles River (Sulzfeld, Germany). The total sample consisted of 64 mice divided in four treatment groups of 16 mice each and two conditions (SHC, CSC). The final number per group was N=8.

Mice were kept under specific pathogen free conditions in a 12h/12h light-dark cycle (lights on at 06:00h winter time; lights on at 07:00h summer time; 22°C; 60% humidity), with free access to tap water and standard mouse diet. Experimental mice were housed in standard polycarbonate mouse cages (16 cm width x 22 cm length x 14 cm height).

Since the CSC paradigm is based on territorial aggression and the establishment of social hierarchies, which are not typically observed in female mice, these were not used in the current study.

2.3 Experimental procedures

Experimental timeline is shown in Figure 2. Mice were housed in groups of four on the day of arrival (day -21) and on day -7 they were individually housed for one week before the start of CSC exposure (day 1).

Treatments were differentiated between prior and during stress exposure, using different combinations of *M. vaccae* or vehicle (Veh, BBS=borate-buffered saline). In summary, the four treatment conditions were the following: BBS prior + BBS during (SHC, N=8; CSC, N=7); BBS prior + *M. vaccae* during (SHC, N=8; CSC, N=8); *M. vaccae* prior + BBS during (SHC, N=8; CSC, N=8); *M. vaccae* prior + *M. vaccae* during (SHC, N=8; CSC, N=8).

Mice received intragastric administration of Veh or *M. vaccae* on days -21, -14, -7, 2, 8 and 15.

To assess anxiety-related behaviors, behavioral tests were performed for SHC and CSC mice: Elevated zero-maze (EZM) on day 19; Open-field/novel object (OF/NO) on day 20; Social preference/avoidance test (SPAT) on day 21.

Body weight was assessed on days -21, -14, -7, 1, 5, 8, 12, 15, 19, 21 and 22.

One mouse of the CSC condition treated with BBS prior + BBS during died during CSC.

All experimental mice were exposed to 21 days of either CSC or SHC condition and euthanized on day 22 between 07:00h and 10:00h a.m. by rapid decapitation following brief CO_2 exposure.

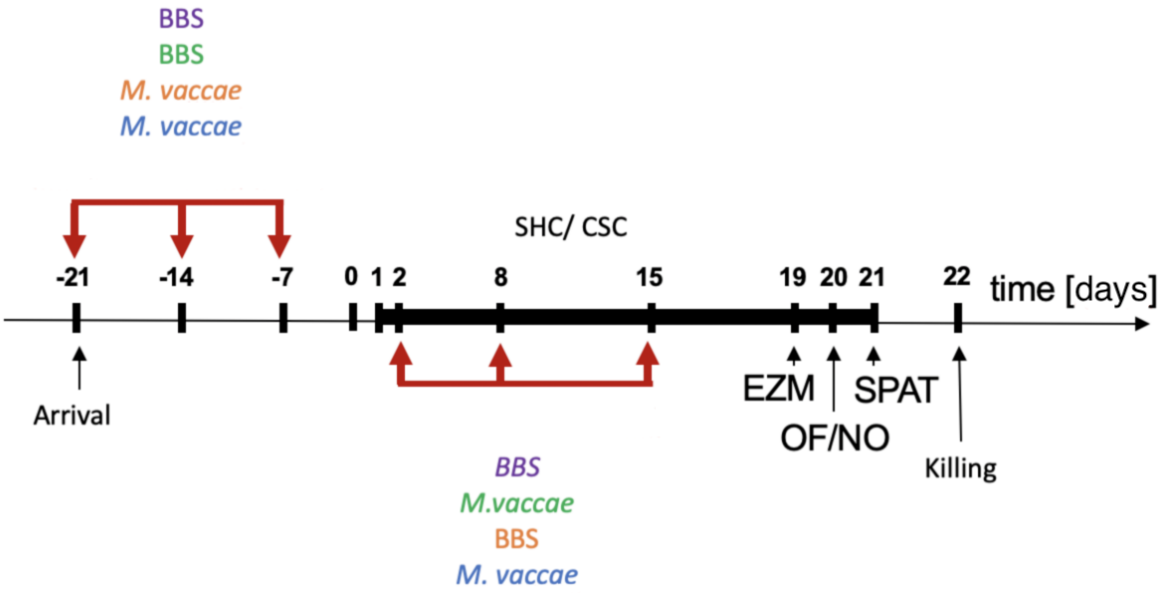

Figure 2 Schematic illustration of the experimental timeline. Male C57BL/6N mice were exposed to the chronic subordinate colony housing (CSC) paradigm or kept as single-housed controls (SHC). *Mycobacterium vaccae* (*M. vaccae*), borate-buffered saline (BBS) or a combination of both was administered before (days -21, -14, -7), and during (days 2, 8, 15) CSC exposure. Mice were euthanized on day 22. EZM = Elevated zero maze; OF/NO = Open field/ novel object test; SPAT = Social preference/ avoidance test.

2.3.1 Chronic subordinate colony (CSC) housing

The CSC paradigm was conducted as described previously (Langgartner et al., 2015; Reber et al., 2016). Briefly, four experimental CSC mice were housed together with a dominant male aggressor mouse for 21 consecutive days to induce chronic psychosocial stress (Figure 3). Male aggressors that started injuring the experimental mice by harmful bites were excluded. To avoid habituation, experimental mice were transferred in the home cage of a new aggressor mouse on days 8 and 15 of the CSC procedure. SHC mice remained undisturbed in their home cages during the whole experiment, except for changing the bedding once a week.

Figure 3 CSC mice (black) showing submissive upright posture towards the resident mouse (white).

2.3.2 Intragastric administration of *M. vaccae*

M. vaccae and Veh were administered using oral gavage needles (Fine Science Tool: # 18060-20, stainless steel, 20 ga x 30 mm). Mice were grabbed by their back: the gavage needle was inserted into the oral cavity and the mouse was allowed to swallow it (Figure 4). In this way, the treatment was infused directly into the stomach of each mouse. Every administration consisted of 100 µl of *M. vaccae* (1 mg/ml) or Veh.

Figure 4 Procedure of intragastric administration. From the left: the mouse is held by its back; the gavage needle is inserted into the mouth of the mouse; the mouse swallows the gavage needle. In this way, the treatment directly reaches the stomach.

2.4 Behavioral assessments and tests

2.4.1 Elevated zero-maze (EZM)

Elevated zero maze represents a modification of the elevated plus maze, with the advantage of lacking the central neutral area (Shepherd et al., 1994).

The test is based on the conflictive tendency of mice to explore novel environments and avoid elevated and open spaces that could potentially expose them to predators.

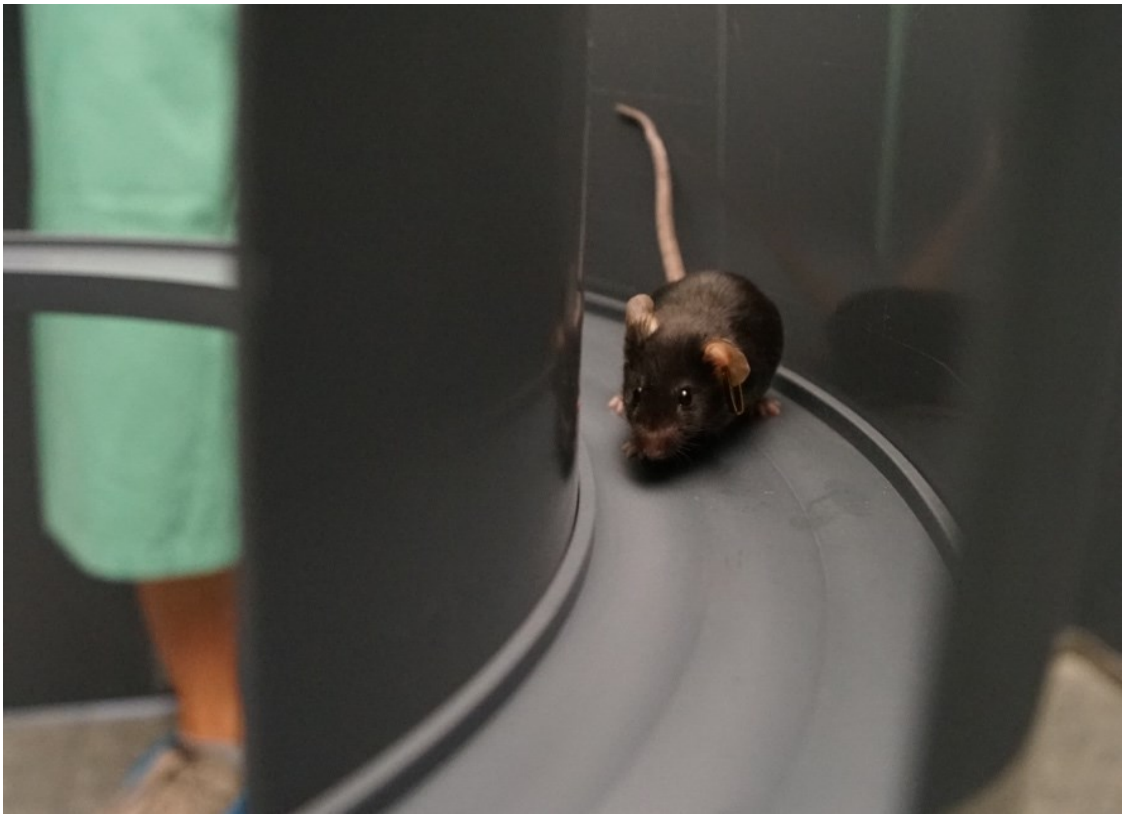

Figure 5 Mouse inside the closed arm of the elevated zero maze

The apparatus consists of two open and two closed arms, elevated 130 cm above the floor, with circular shape (Figure 6). Light intensity was set to 100 lux for the open arms and 45 lux for closed arms. As a measure of anxiety, the time spent in exploring the open arms compared to the total time spent in the maze was used. EthoVision XT (Version v11.5.1022, Noldus Information Technology, Wageningen, Netherlands) was used to record the parameters.

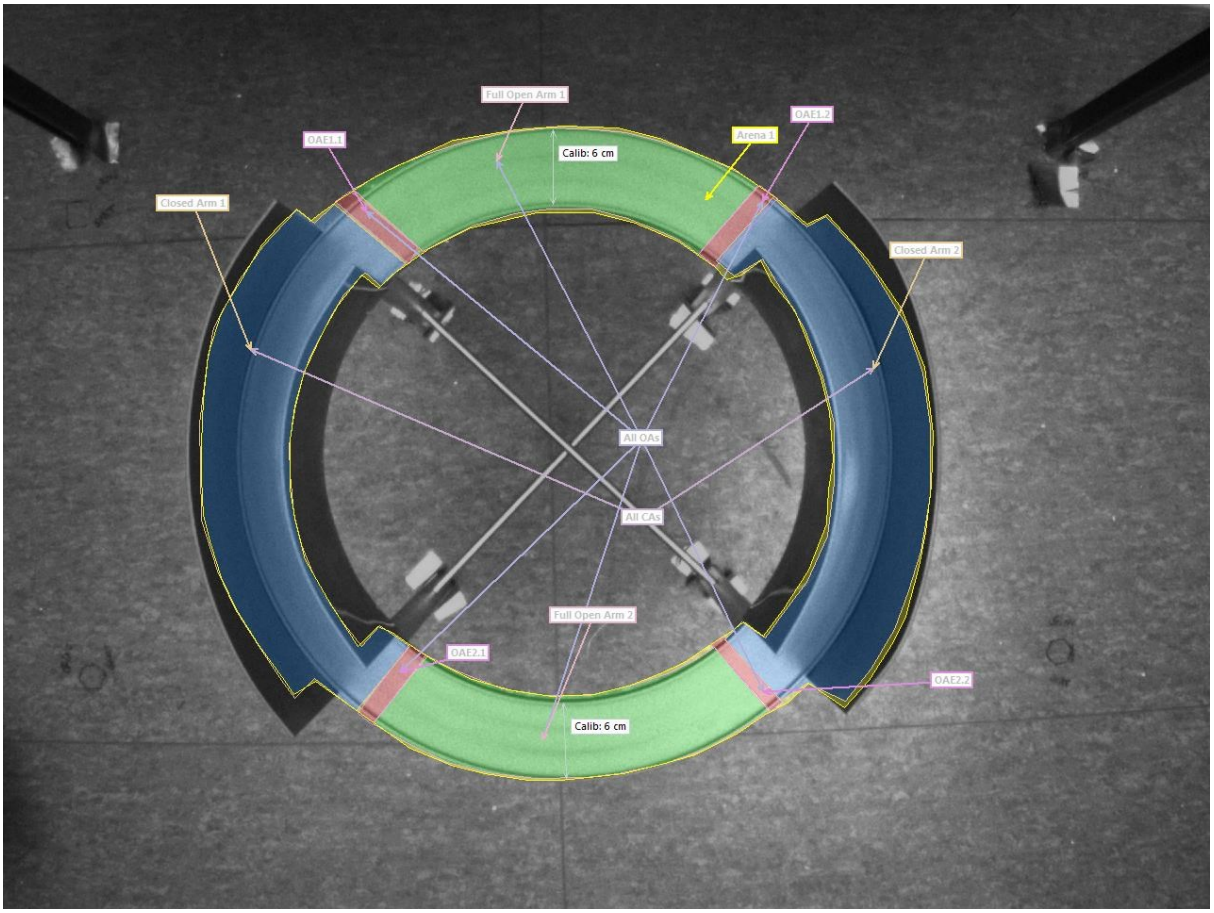

Figure 6 EZM arena with areas: Closed arms (blue), Full open arms (green), Open arm entries (red).

2.4.2 Open-field/novel object (OF/NO) test

CSC effects on anxiety-related behaviors were assessed exposing the experimental mice to the open-field/novel object (OF/NO) test on day 20 of the CSC paradigm (Heredia et al., 2014). Briefly, the arena (45 cm length × 27 cm width × 27 cm height) was subdivided into an inner zone (27 cm × 9 cm), an outer zone and four corners (Figure 8). In the OF test, mice were placed into the inner zone and could explore the arena for 5 min. After 5 min of OF exploration, a plastic round object (diameter: 3.5 cm; height: 1.5 cm), which represents the novel object (NO), was placed in the middle of the inner zone. The mouse was allowed to explore the arena containing the unfamiliar object for 5 min.

Figure 7 Mouse during open field (left) and novel object (right) tests.

In the OF test, the distance moved was used to assess general locomotion during the test. In the NO test, the number of object explorations, the time spent in the corners of the arena and the distance moved were used as parameters of anxiety-related behavior. EthoVision XT (Version v11.5.1022, Noldus Information Technology) was used to record the parameters.

The test was performed between 07:00 a.m. and 10:00 a.m. under white light conditions (~350 lux). The arena was cleaned thoroughly before each trial.

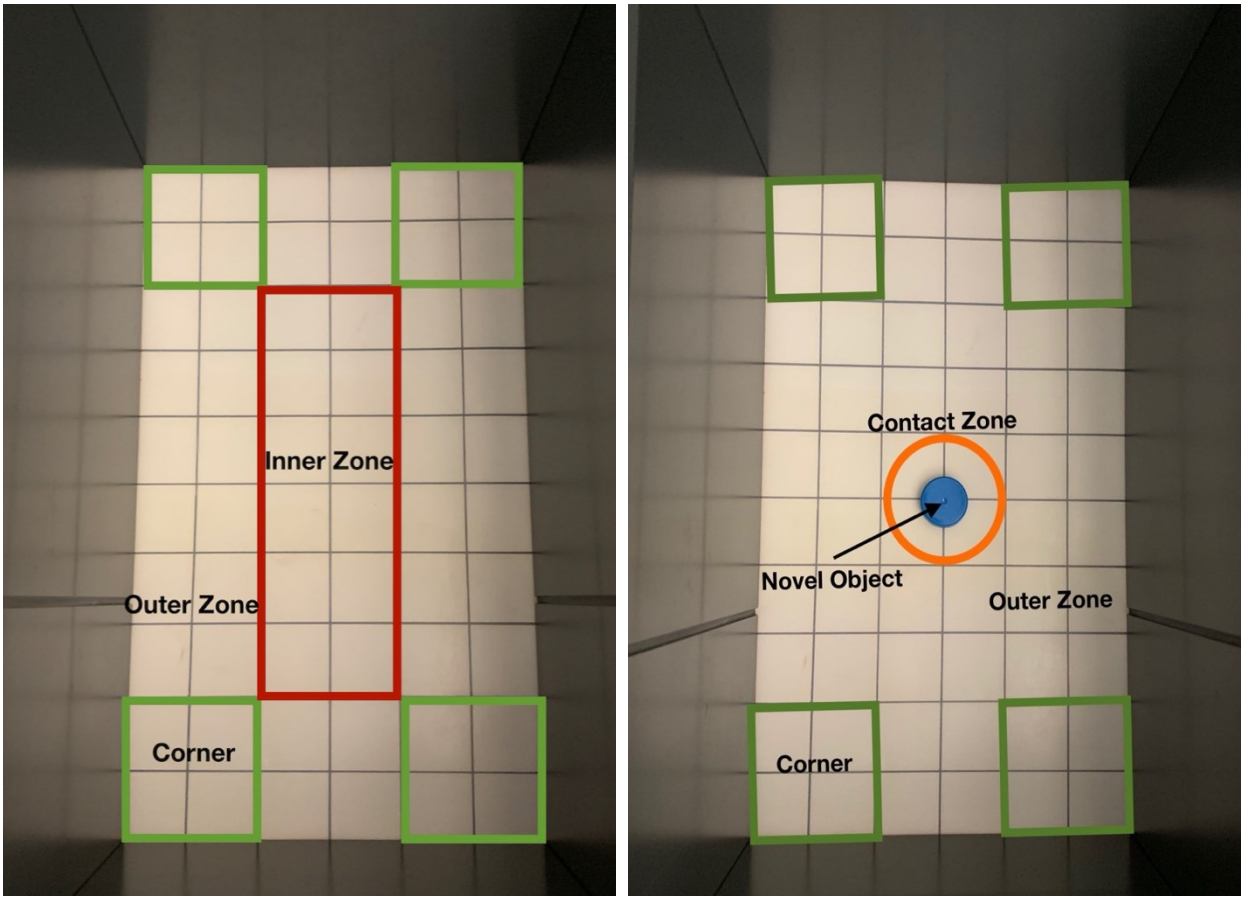

Figure 8 Open Field/Novel Object arena with areas: Corners, Outer zone NO, Outer zone OF, Open field OF, Contact zone NO.

2.4.3 Social preference/avoidance test (SPAT)

Mice were exposed to the social preference/avoidance test (SPAT) on day 21 to assess the effects of *M. vaccae* injections and/or CSC exposure on general and social anxiety-related behaviors. Briefly, the experimental mouse was placed into the SPAT box (length: 45 cm; width: 27 cm; height: 27 cm; light intensity: 20 lux) for 30 s to habituate to the unfamiliar environment before a small empty wire mesh cage (length: 8.5 cm; width: 7.5 cm; height: 6.5 cm) was introduced into the SPAT box for 150 s.

Figure 9 Experimental mouse interacting with an unfamiliar male mouse during the social preference/avoidance test.

Afterwards, the empty cage was exchanged with an identical cage containing an unfamiliar male mouse for another 150 s (Figure 9). EthoVision XT (Version v11.5.1022, Noldus Information Technology) was used to record total distance moved and time spent in the 2.25-cm broad direct contact zone around the wire mesh cage during both 150 s trials. The box was cleaned thoroughly with water before every test. The SPAT took place between 07:00 a.m. and 11:00 a.m.

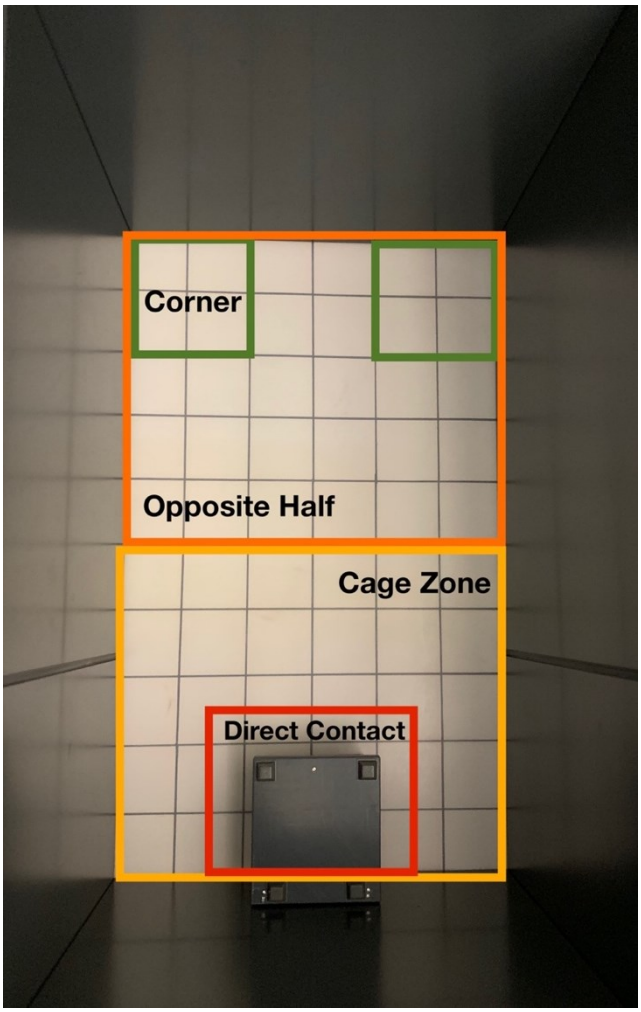

Figure 10 SPAT arena with areas: Corners (green), Opposite half (orange), Cage zone (yellow), Direct contact (red).

2.5 Physiological and immunological parameters

On day 22 all mice were rapidly euthanized by decapitation following brief CO_2 exposure between 07:00h and 10:00h.

After decapitation and trunk blood collection, spleen, adrenal glands, and thymus were harvested for weighing and further analyzed.

2.5.1 Body weight

SHC and CSC mice were weighed on day 1 before the start of the CSC paradigm and on day 22 before decapitation. Body weight gain was calculated according to the following formula: body weight on day 22- body weight on day 1.

2.5.2 Trunk blood sampling

Trunk blood was collected in EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) and stored on ice until centrifugation at 4℃ (5000 g, 10 min). Plasma samples were stored at –20℃ until analysis using an enzyme-linked immunosorbent assay (ELISA) kit for Corticosterone (CORT) detection.

2.5.3 Determination of organs weight

Following euthanasia, organs were removed, pruned of fat and weighed. Specifically, left and right adrenal glands, spleen and thymus of each animal were weighed.

2.5.4 Corticosterone ELISA

After 1:10 dilution of 10µl of plasma in Dulbecco's phosphate buffer solution (dPBS; Gibco life Technologies, United Kingdom), plasma corticosterone (CORT) levels were measured using a commercially available enzyme linked immunosorbent assay (ELISA; IBL international; Hamburg, Germany).

2.5.5 Determination of GC sensitivity in splenocytes

After removal, the spleens were weighed and stored in ice cold Hanks' balanced salt solution (HBSS; Sigma-Aldrich, United Kingdom) until splenocytes isolation.

Splenocytes isolation was performed as previously described (Förtsch et al., 2017; Reber et al., 2007). Briefly, the spleens were smashed through a 70µm nylon cell strainer (Corning, USA) into a 50ml centrifugation tube (Falcon, USA) to obtain a single-cell suspension. Erythrocytes were removed by adding Ammonium-Chloride-Potassium (ACK) lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA) for 2 minutes followed by the addition of HBSS + 10 % FCS (Gibco life Technologies, United Kingdom) to stop the lysis reaction. After centrifugation and a washing step, the cell pellet was resuspended in HBSS and filtered again through a cell strainer. After another centrifugation step, the suspension was adjusted to $5x10^6$ cells per ml in RPMI+ medium (RPMI-1640 [Sigma-Aldrich, United Kingdom] + 10% FCS + 1% penicillin + streptomycin [Pen Strep, Life Technologies]). The isolated splenocytes were counted by using an automated cell counter (TC-20, BioRad Laboratories, München, Germany).

Isolated splenocytes were stimulated *in vitro* in a flat-bottom 96-well plate (Falcon, USA). With *E. coli*-derived lipopolysaccharide (LPS; Sigma Aldrich, United Kingdom; 1µg/ml). To assess whether splenocytes were sensitive or resistant to the suppressive effects of glucocorticoids, unstimulated and LPS-stimulated splenocytes were treated with various CORT concentrations

(Sigma-Aldrich, United Kingdom; final concentrations were 0, 0.1 and 5 µM respectively), diluted in 95% ethanol. Final volume was 100µl per well. After 24 hours incubation (37°C, 5% CO_2), cell proliferation was measured by using an MTS assay (20 µl/ well; ab197010, Abcam). Following 3 hours incubation, cell viability was measured by using an ELISA plate reader (Fluostar Optima, BMG Labtech, Offenburg, Germany). The absorbance, expressed as optical density (OD), was determined at 450 nm.

2.5.6 Bite score assessment

Glucocorticoid resistance in splenocytes, splenic immune activation and splenomegaly have been shown to depend on the extent of bite wounds received. For this reason, the severity of dermal and subdermal bite wounds has been analyzed and a bite score was assessed as previously described (Foertsch et al., 2017).

The procedure consists in fur removal of CSC mice after decapitation and taking pictures of fur and body to assess dermal and subdermal damages. Then, the photographs were overlaid with a grid of 20 squares to contain all body and skin: every square was scored separately. After the scoring, the combination of all squares score showed the total bite ranging, which ranged from a minimum value of 0 to a maximum value of 300, according to size, severity and purulence.

2.6 Data analysis and statistics

For statistical analysis, the software package IBM SPSS statistics (version 25.0; IBM Corporation, Armonk, NY, United States) was used.

To test for normal distribution of all acquired data sets, the Kolmogorov-Smirnov test using Lilliefors' correction was employed. Outliers in normally distributed data sets were identified using Grubbs' test and were excluded from further analysis.

Normally distributed data sets were further analyzed using two-way ANOVA (two factors, two or more independent samples). In case of repeated measures, a linear mixed model (LMM) approach was used. In both cases, *post-hoc* analysis using Bonferroni pairwise comparison was used, in case a significant main effect was found.

Non-normally distributed data sets were analyzed using non-parametric statistics, i.e. Mann-Whitney U test (MWU; one factor, two independent samples), Wilcoxon test (one factor, two dependent samples) and Friedman's ANOVA (one factor, more than two dependent samples). The graphs were made using the software package SigmaPlot (version 13.0; Systat Software Inc., San Jose, CA, United States). Bars represent normally distributed data (mean + SEM), while non-normally distributed data are represented as box plots. Solid lines represent the median and dashed lines represent the mean for each data set. 10^{th}, 25^{th} 75^{th} and 90^{th} percentiles are shown, as well as possible outliers beyond the percentiles represented by closed circles. The level of significance was set at $p \leq 0.05$.

CHAPTER

Results 3

3.1 Effects of *M. vaccae* on body and organs weight

3.1.1 Body weight

Body weight was determined on days 1 and 22 of CSC exposure and the delta body weight development was calculated (Figure 11). Body weight gain was increased in CSC vs. SHC mice in the BBS prior + *M.vaccae* during treated group only (Fig. 11; $F(Stress)_{1,62} = 8.174$; $p = 0.006$; Bonferroni: $p = 0.048$).

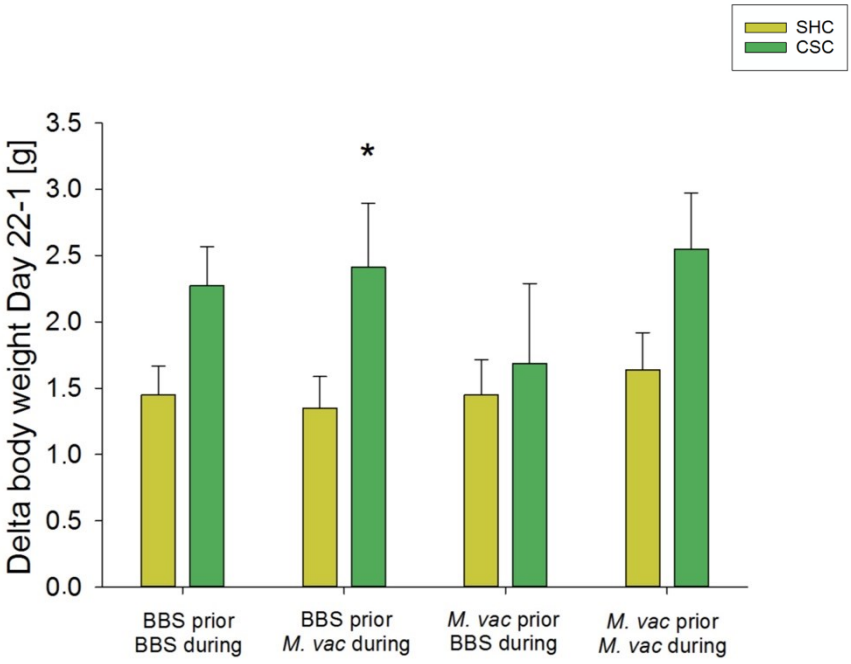

Figure 11 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on delta body weight between day 22 and day 1.
The delta body weight was significantly increased in CSC vs.SHC mice in the BBS prior + *M. vaccae* during treatment only.
Parametric data are given as mean + SEM. SHC: single-housed controls; CSC: chronic subordinate colony; *M. vac: Mycobacterium vaccae*; BBS: borate-buffered saline; SEM: standard error of the mean. For each group N = 8, except for CSC BBS prior + BBS during N = 7.
* $p \leq 0.05$ vs. respective SHC group.

SHC: single-housed controls; CSC: chronic subordinate colony; *M. vac*: *Mycobacterium vaccae*; BBS: borate-buffered saline

3.1.2 Adrenal glands

Relative adrenal weight was increased in CSC vs. SHC mice in all groups (Fig. 12; MWU; $p <$ 0.001 in all groups respectively), except for the *M. vaccae* prior + BBS during group.

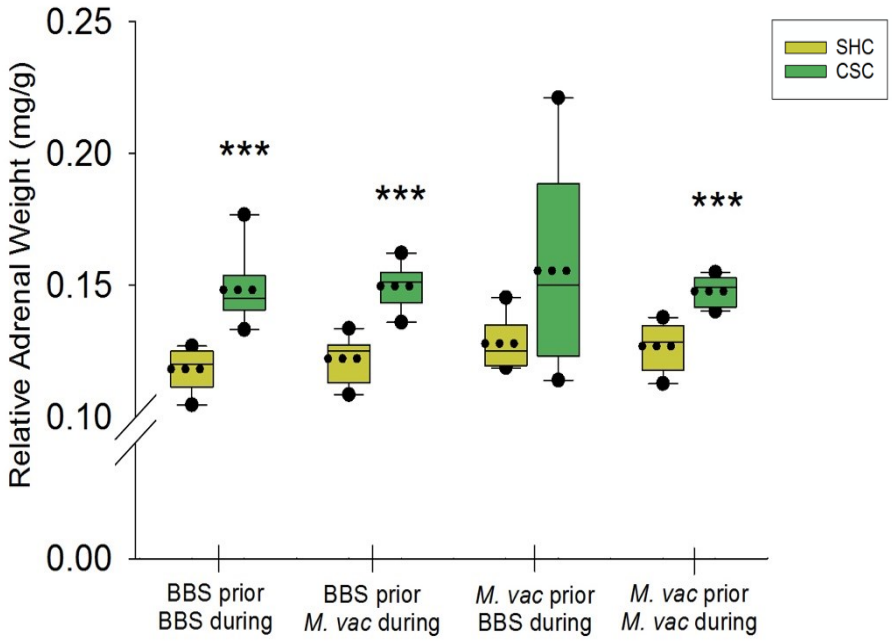

Figure 12 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on relative adrenal weight.
Relative adrenal weight was significantly increased in CSC animals compared to their respective SHC group, except for *M. vaccae* prior + BBS during group.
Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. For each group N = 8, except for CSC BBS prior + BBS during N = 7.
*** for $p \leq 0.001$ vs. respective SHC group.

SHC: single-housed controls; CSC: chronic subordinate colony; *M. vac*: *Mycobacterium vaccae*; BBS: borate-buffered saline

3.1.3 Thymus

Relative thymus weight was decreased in CSC vs. SHC mice in all groups (Fig. 13; $F(\text{stress})_{1,55} = 17.766$, $p < 0.001$; Bonferroni: BBS prior + BBS during $p = 0.016$, BBS prior + *M. vaccae* during $p = 0.036$, *M. vaccae* prior + BBS during $p = 0.004$) except for the *M. vaccae* prior + *M. vaccae* during group. Furthermore, CSC mice in the *M. vaccae* prior + *M. vaccae* during group showed an increased relative thymus weight when compared to respective *M. vaccae* prior + BBS during group (Fig. 13; $F(\textit{M. vaccae})_{3,55} = 4.311$, $p = 0.008$; Bonferroni: $p = 0.006$).

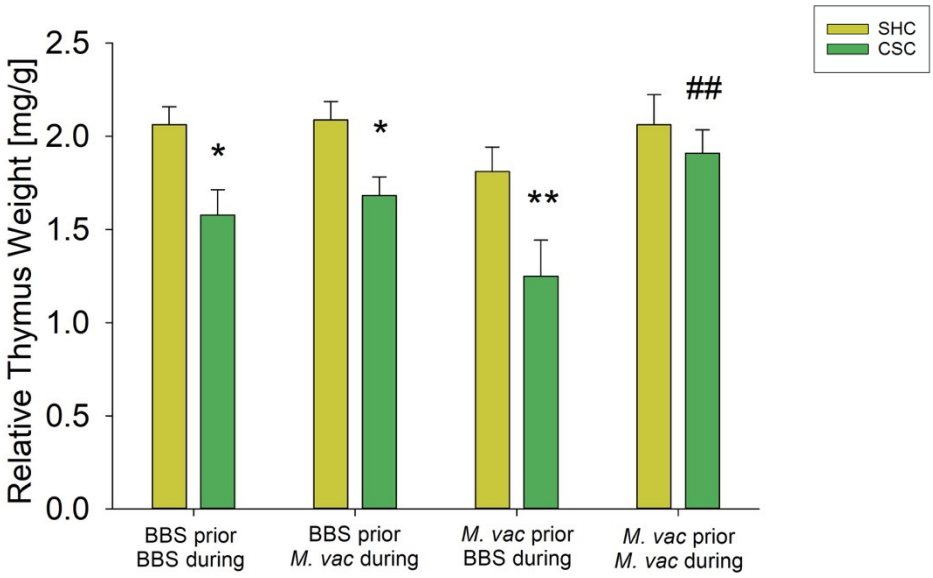

Figure 13 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on relative thymus weight.
Thymus weight was signficantly decreased in all CSC vs. SHC mice except for *M. vaccae* prior + *M. vaccae* during. CSC mice in the latter group had significantly increased relative thymus weight compared to respective *M. vaccae* prior + BBS during group.
Parametric data are given as mean + SEM. For each group N = 8, except for CSC BBS prior + BBS during N = 7.
* for $p \leq 0.05$ and ** for $p \leq 0.01$ vs. respective SHC group.
for $p \leq 0.01$ vs. respective *M. vaccae* prior + BBS during group.

SHC: single-housed controls; CSC: chronic subordinate colony; *M. vac: Mycobacterium vaccae*; BBS: borate-buffered saline; SEM: standard error of the mean.

3.1.4 Spleen

Absolute spleen weight was significantly increased in CSC vs. SHC mice in the *M. vaccae* prior + BBS during group (Fig. 14; MWU; p=0.010) and in the *M. vaccae* prior + *M. vaccae* during group (Fig. 14; MWU; p=0.005).

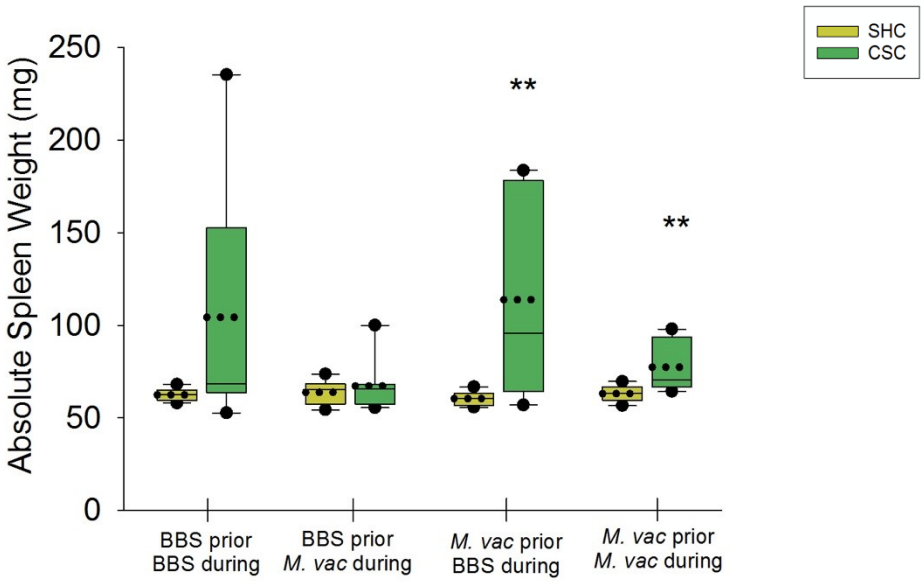

Figure 14 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on absolute spleen weight.
Absolute spleen weight was increased in CSC vs. SHC mice in *M. vaccae* prior + BBS during and *M. vaccae* prior + *M. vaccae* during treatments.
Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. For each group N=8, except CSC BBS prior + BBS during N=7.
** for p≤0.01 vs. respective SHC group.

SHC: single-housed controls; CSC: chronic subordinate colony; *M. vac*: *Mycobacterium vaccae*; BBS: borate-buffered saline

3.2 Administration of *M. vaccae* and CSC-induced systemic inflammation

3.2.1 Glucocorticoids resistance

Cell viability was significantly increased in CSC vs. SHC mice in the *M. vaccae* prior + BBS during group (Fig. 15; MWU; $p = 0.028$) in the basal condition, and in the BBS prior + *M. vaccae* during (Fig. 15; MWU; $p = 0.028$), in the *M. vaccae* prior + BBS during (Fig. 15; MWU; $p=0.002$) and in the *M. vaccae* prior + *M. vaccae* during group (Fig. 15; MWU; $p=0.001$) in the LPS-stimulated condition. Cell viability in LPS-stimulated vs. basal condition was increased in all groups (Fig. 15; Wilcoxon; $p < 0.05$ in all groups respectively).

The delta cell viability (LPS minus basal, 5 µM CORT) was significantly increased in CSC vs. SHC in the BBS prior + BBS during (Fig. 16A; MWU, $p = 0.001$), in the *M. vaccae* prior + BBS during (Fig. 16A; MWU, $p = 0.021$) and in the *M.va*ccae prior + *M. vaccae* during group (Fig. 16A; MWU, $p = 0.050$). Furthermore, the 3 SHC groups that received *M. vaccae* showed increased delta cell viability (LPS minus basal, 5 µM CORT) compared to SHC BBS prior + BBS during group (Fig. 16A; Kruskal-Wallis; BBS prior + *M.vaccae* during: $p = 0.046$; *M. vaccae* prior + BBS during: $p = 0.012$; *M. vaccae* prior + *M. vaccae* during: $p = 0.003$, all vs. respective BBS prior + BBS during group).

Delta cell viability in the presence of CORT = 5µM was significantly reduced in SHC (Fig. 16B; Friedman: BBS prior + BBS during, BBS prior + *M. vaccae* during, *M. vaccae* prior + BBS during: $X^2_{(2,8)} = 16.000$; $p < 0.001$; *M. vaccae* prior + *M. vaccae* during: $X^2_{(2,8)} = 14.250$; $p = 0.001$) and in CSC (Fig. 16B; Friedman: BBS prior + *M. vaccae* during: $X^2_{(2,7)} = 14.000$; $p = 0.001$; *M. vaccae* prior + BBS during: $X^2_{(2,8)} = 14.250$; $p = 0.001$; *M. vaccae* prior + *M. vaccae* during: $X^2_{(2,8)} = 16.000$; $p < 0.001$) compared to respective 0µM CORT condition (set to 100%) groups. In addition, delta cell viability in the presence of CORT = 5µM (in % of CORT = 0µM set to 100%) was significantly increased in CSC vs. SHC in the BBS prior + BBS during (Fig.

16B; MWU; $p = 0.002$) and in the *M. vaccae* prior + BBS during group (Fig. 16B MWU; $p = 0.028$).

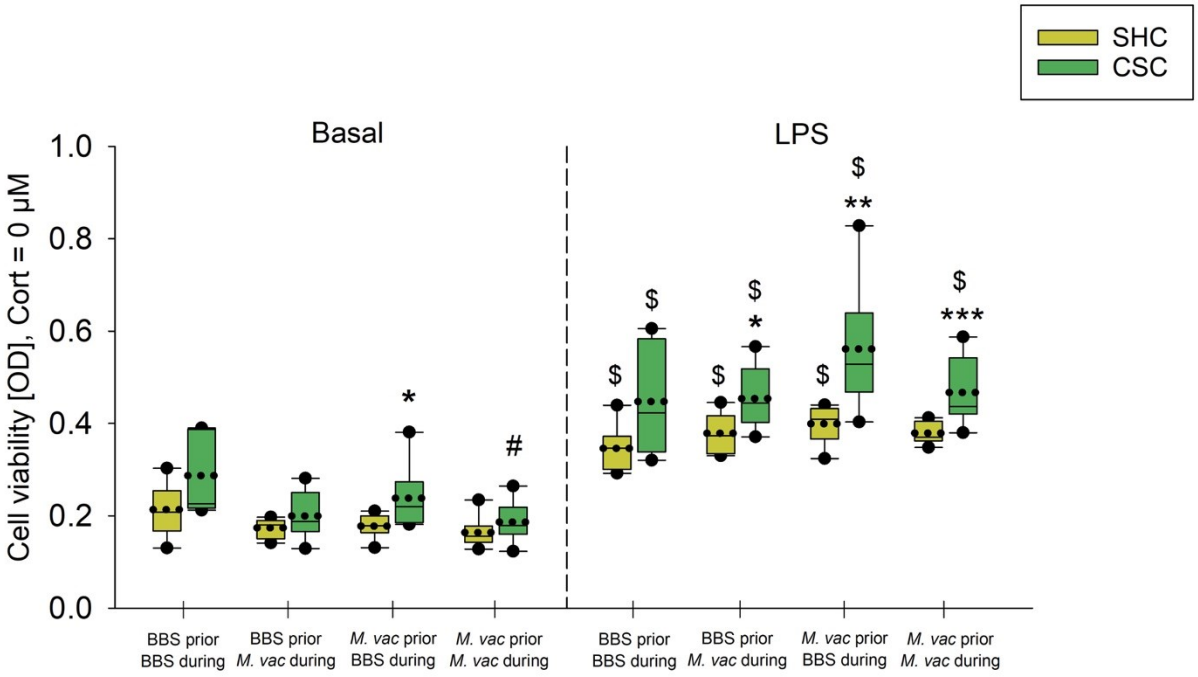

Figure 15 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on splenocyte cell viability in basal vs. LPS-stimulated condition.
Cell viability was increased in CSC vs. SHC mice of *M. vaccae* prior + BBS during group in the basal condition, as well as of BBS prior + *M. vaccae* during, *M. vaccae* prior + BBS during and *M. vaccae* prior + *M. vaccae* during group in the LPS-stimulated condition. All LPS-treated groups have significantly increased cell viability when compared to respective groups in basal condition.
Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. For each group N = 8, except CSC BBS prior BBS during N = 7.
* for $p \leq 0.05$; ** for $p \leq 0.01$ and *** for $p \leq 0.001$ vs. respective SHC group.
# for $p \leq 0.05$ vs. respective BBS prior + BBS during group.
$ for $p \leq 0.05$ vs. respective group in basal condition.

SHC: single-housed controls; CSC: chronic subordinate colony; *M. vac*: *Mycobacterium vaccae*; BBS: borate-buffered saline; LPS: lipopolysaccharide.

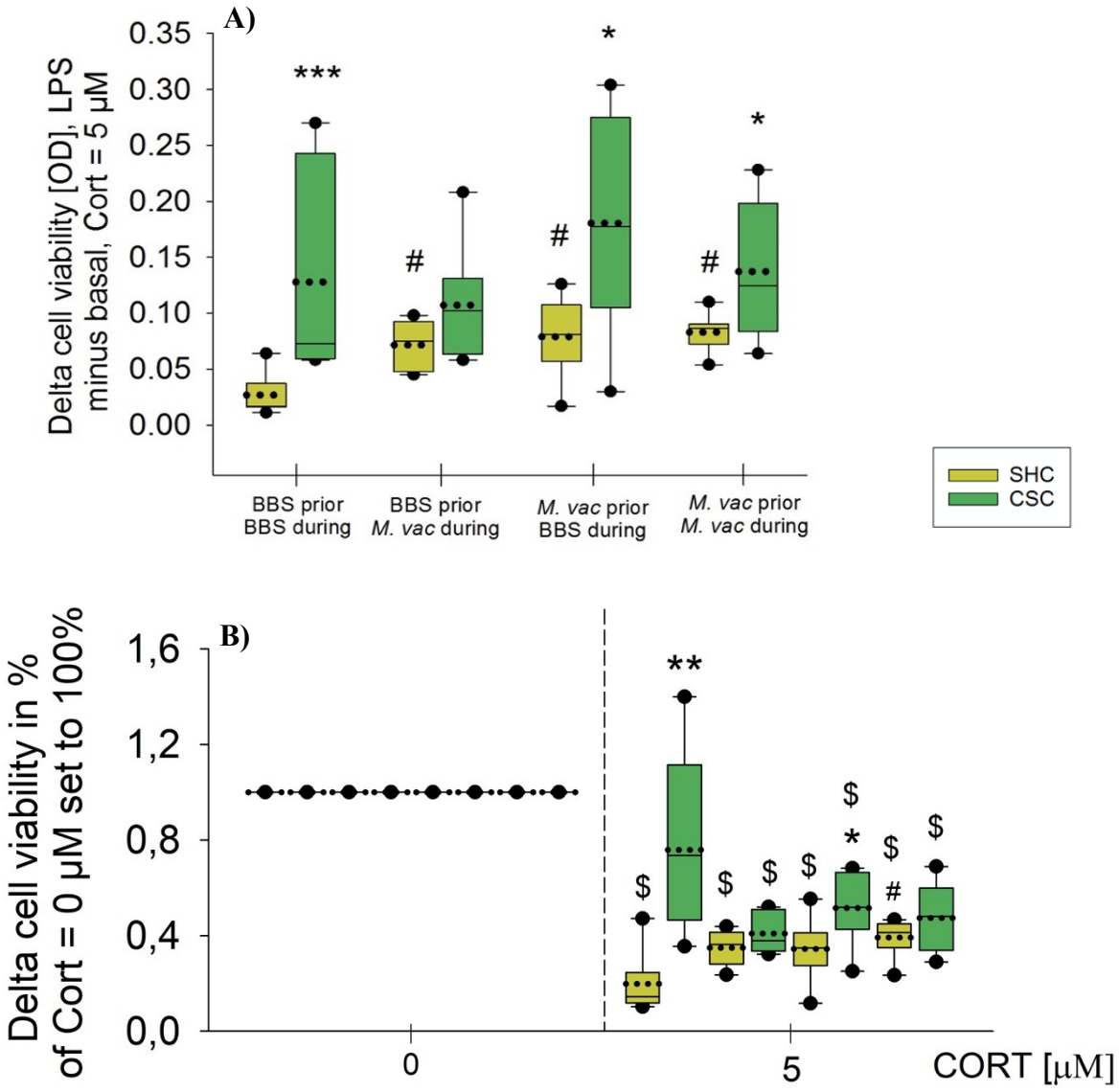

Figure 16 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on cell viability of splenocytes was measured after 24 h of incubation in the absence of (basal) or with lipopolysaccharide (LPS) at different CORT concentrations.

A) Delta cell viability of splenocytes (LPS minus basal) at CORT = 5µM condition. B) Delta cell viability (in % of CORT = 0µM set to 100%) at CORT = 0µM and CORT = 5µM conditions.

Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. For each group N=8, except CSC Veh N=7.

* for p≤0.05; ** for p≤0.01 and *** for p≤0.001 vs. respective SHC group.

for p≤0.05 vs. respective BBS prior + BBS during group.

$ for p≤0.05 vs. respective group in 0µM condition.

SHC: single-housed controls; CSC: chronic subordinate colony; *M. vac: Mycobacterium vaccae*; BBS: borate-buffered saline; LPS: lipopolysaccharide.

3.2.2 Bitescore

No significant difference was observed in the bitescore of any of the experimental groups (Figure 17).

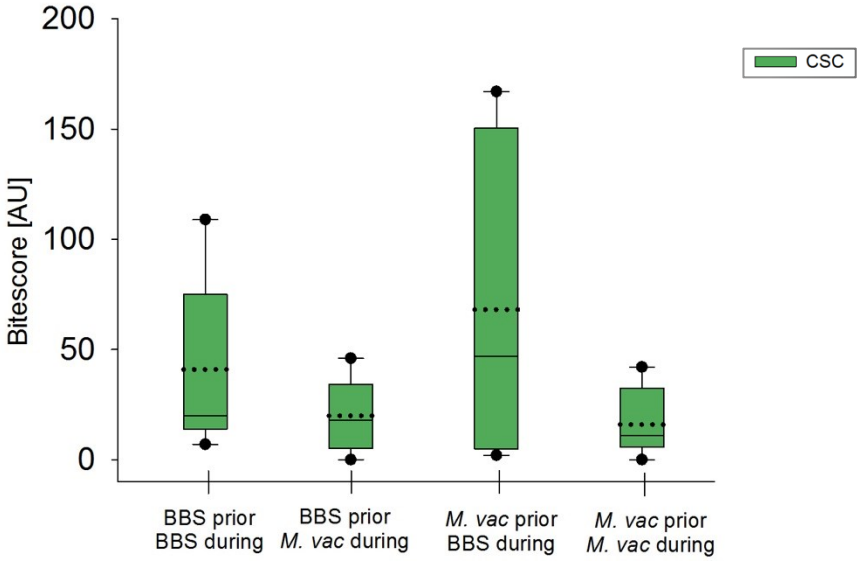

Figure 17 Bitescore.
Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown.For each group N=8, except CSC Veh N=7.
M. vac: *Mycobacterium vaccae*; BBS: borate-buffered saline

3.2.3 Correlation analysis: spleen weight vs. bitescore

Development of splenomegaly is highly dependent on the bite wounds that the animals received during CSC exposure (Foertsch et al., 2017). Statistical analysis did not show any difference in the spleen weight among the groups.

To assess whether the results were in line with previous studies, a correlation analysis between spleen weight and bitescore was made for all groups. A positive correlation was found in all CSC groups (Fig. 18; BBS prior+BBS during r=0.893, *p*=0.007; BBS prior+*M. vaccae* during r=0.731, *p*=0.04; *M. vaccae* prior+BBS during r=0.893, *p*=0.007; *M. vaccae* prior+*M. vaccae* during r=0.731, *p*=0.04).

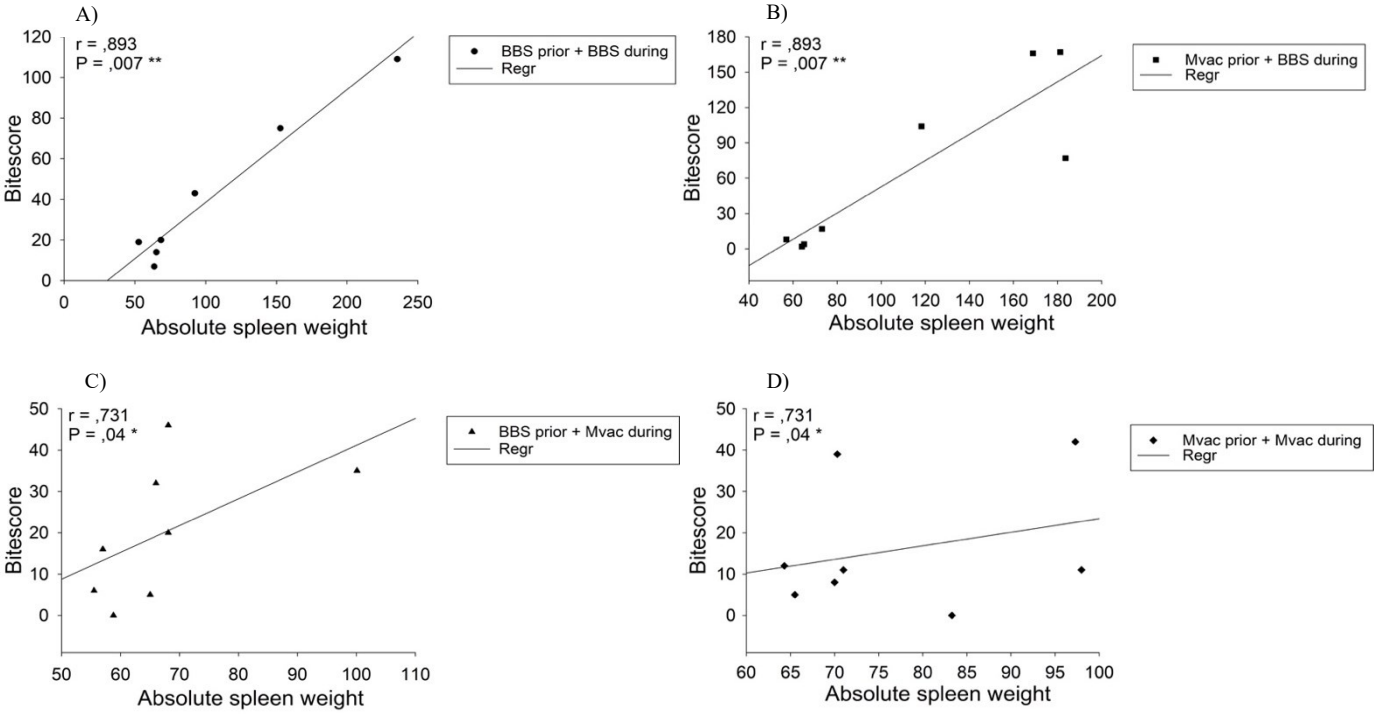

Figure 18 Correlation analysis of bitescore and spleen weight.
A) Bitescore and spleen weight positively correlate with each other for BBS prior + BBS during mice (correlation coefficient = 0.893, p = 0.007; Spearman-Rho correlation; N=7). C) Bitescore and spleen weight positively correlate with each other for BBS prior + *M.vaccae* during-trated mice (correlation coefficient = 0.731, p = 0.04; Spearman-Rho correlation; N=8). B) Bitescore and spleen weight positively correlate with each other for *M. vaccae* prior + BBS during mice (correlation coefficient = 0.893, p = 0.007; Speraman-Rho correlation; N=8). D) Bitescore and spleen weight positively correlate with each other for *M. vaccae* combined-treated mice (correlation coefficient = 0.731, p = 0.04; Spearman-Rho correlation; N=8).

M. vac: *Mycobacterium vaccae;* Veh: Vehicle

3.2.4 Plasma CORT

Plasma CORT concentration was significantly increased in CSC vs. SHC mice in the *M. vaccae* prior + BBS during group (Fig. 19; MWU; $p < 0.001$). Furthermore, CORT concentration was also increased in the SHC BBS prior + BBS during group (Kruskal-Wallis; $p=0.014$) when compared to the respective *M. vaccae* prior + BBS during.

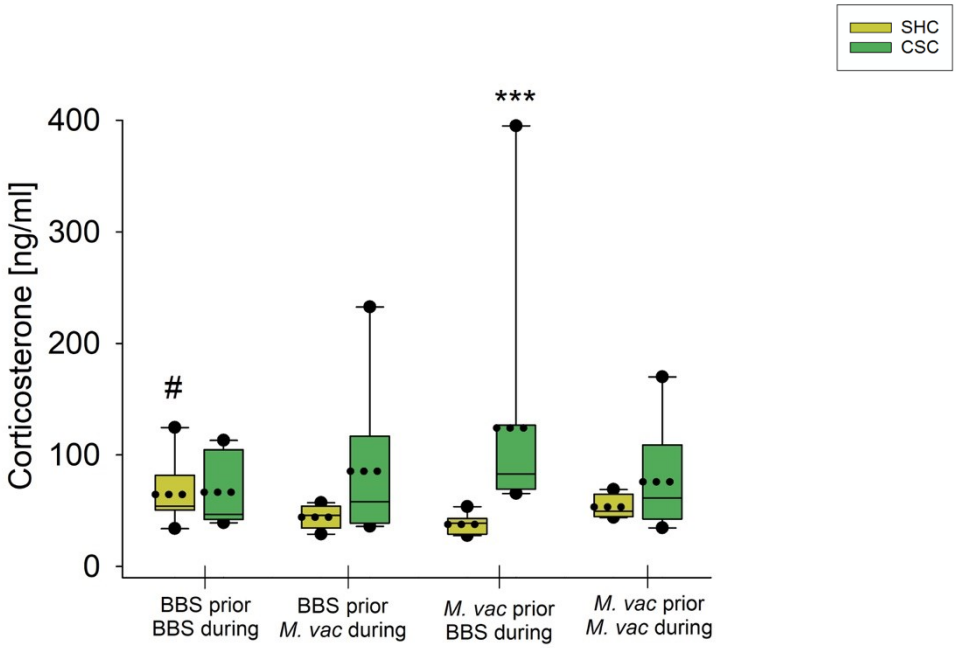

Figure 19 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on plasma corticosterone (CORT) concentration.
Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. For each group N=8, except CSC Veh N=7.
*** for p≤0.001 vs. respective SHC group.
# for p≤0.05 vs. respective *M.vaccae* prior + BBS during group.

SHC: single-housed controls; CSC: chronic subordinate colony; *M. vac*: *Mycobacterium vaccae*; BBS: borate-buffered saline; CORT: corticosterone.

3.3 Effects of intragastric administration of *M. vaccae* on CSC-induced anxiety-related behavior

In this study, general anxiety and social anxiety-related behaviors were assessed in the experimental mice.

3.3.1 General anxiety

•EZM

Mice were tested for general anxiety-related behavior on the EZM test on day 19 of the experimental protocol (Figure 2).

Total distance moved in the EZM was not affected neither by CSC nor by any of the treatments (Figure 20A). On the other hand, mice in the CSC BBS prior + *M. vaccae* during group showed an increased percent of time spent in open arms (Fig. 20B; Kruskal-Wallis; p=0.006) when compared to the respective *M. vaccae* prior + BBS during.

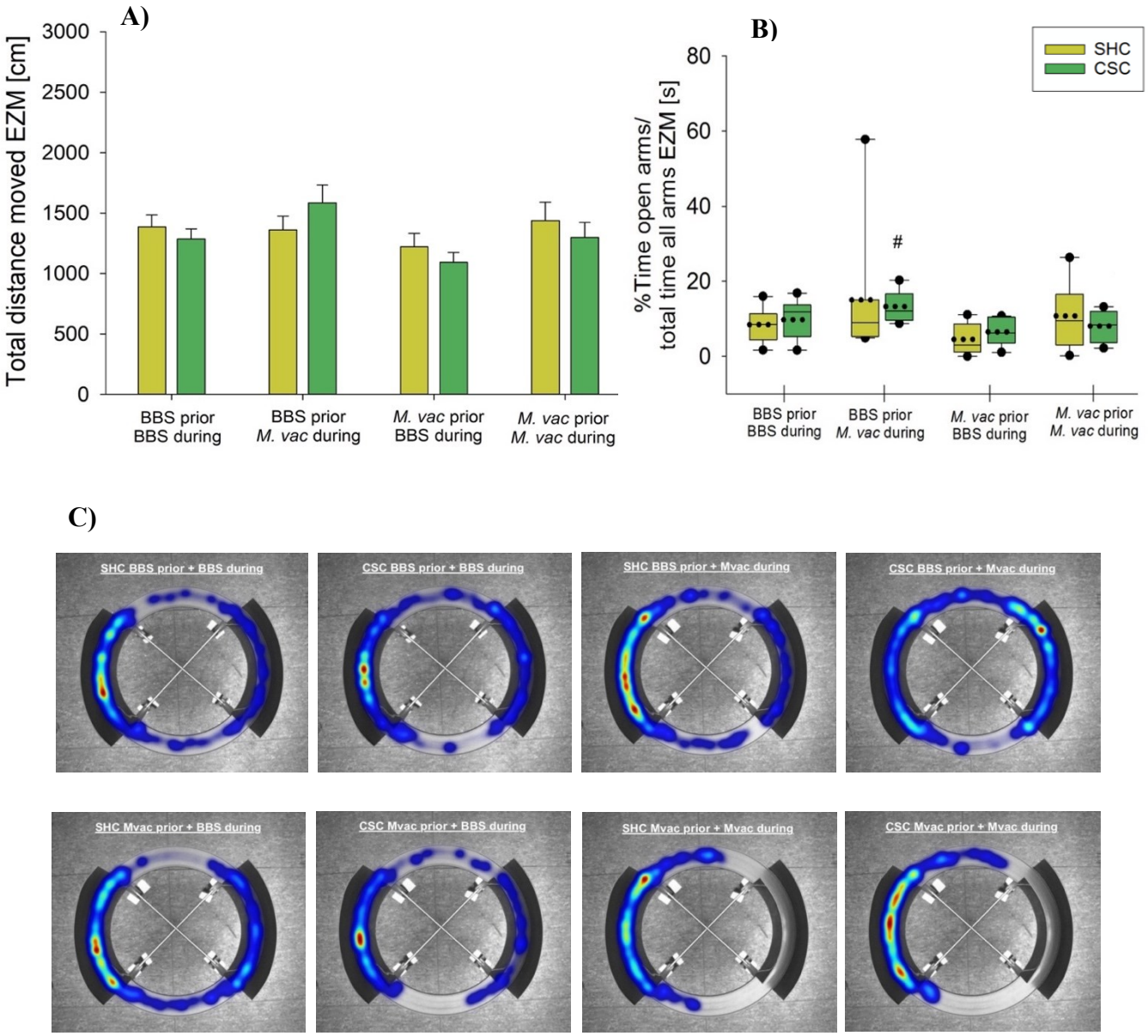

Figure 20 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on EZM. A)Total distance moved EZM (cm); B) % time in open arms/total time all arms EZM (s); C) Time spent in the different areas is shown in heatmaps: red colour represent more time spent in the respective area. Parametric data are presented as mean + SEM. Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. For each group N=8, except CSC Veh N=7.
for $p \leq 0.05$ vs. respective BBS prior + BBS during group.

EZM=elevated zero maze; BBS=borate buffered saline; *M. vac= Mycobaterium vaccae*

•OF/NO

Mice were tested for general anxiety-related behavior on the OF/NO test on day 20 of the experimental protocol (Figure 2).

Mice showed a significantly increased total distance moved during the OF test in CSC BBS prior + *M. vaccae* during group (Fig. 21A; F(*M. vaccae*) $_{3,55}$ = 3.010, p = 0.038; Bonferroni: p = 0.005) when compared to respective BBS prior + BBS during group. Total distance moved during NO test was significantly lower in CSC vs. SHC in the BBS prior + BBS during group (Fig. 21B; MWU; p = 0.021).

Nose point entries in contact zone during the NO test were significantly lower in CSC vs. SHC in the BBS prior + BBS during (Fig. 21C; F(stress) $_{1,55}$ = 10.421, p = 0.002; Bonferroni: p = 0.003) and in the BBS prior + *M. vaccae* during group (Fig. 21C; F(stress) $_{1,55}$ = 10.421, p = 0.002; Bonferroni*:* p = 0.010). Moreover, the number of entries in contact zone with the novel object was increased in the SHC BBS prior + BBS during group (Fig. 21C; F(*M. vaccae*) $_{3,55}$ = 3.090, p = 0.034; Bonferroni: p = 0.021) and in the SHC BBS prior + *M. vaccae* during group (Fig. 21C; F(*M. vaccae*) $_{3,55}$ = 3.090, p = 0.034; Bonferroni: p = 0.012) when compared to the respective *M. vaccae* prior + BBS during group.

Time spent in corners during the NO test was significantly increased in CSC vs. SHC mice in the BBS prior + BBS during group (Fig. 21D; F(stress) $_{1,55}$ = 7.949, p = 0.007; Bonferroni: p = 0.012) and in the BBS prior + *M.vaccae* during group (Fig. 21D; F(stress) $_{1,55}$ = 7.949*,* p = 0.007; Bonferroni: p = 0.025).

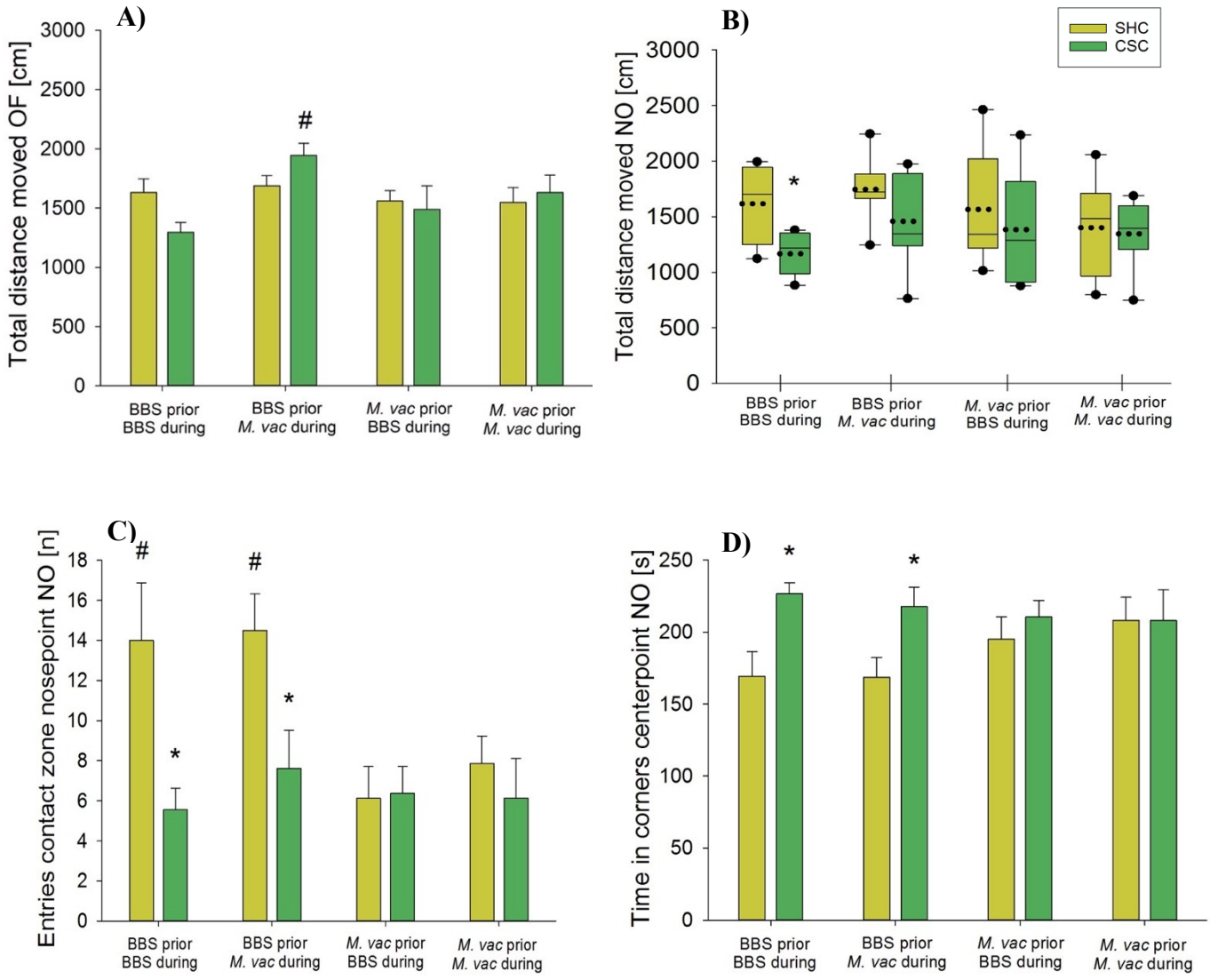

Figure 21 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on OF/NO.

A)Total distance moved is increased in CSC BBS prior+*M.vaccae* during mice when compared to respective Veh group. (# vs BBS prior + BBS during). B) Total distance moved is decreased in CSC Veh mice during novel object. C) CSC Veh and BBS prior + *M. vaccae* during mice show a lower number of entries in contact zone during novel object when compared to respectivre SHC. The SHC groups of these treatments have a higher number of entries when compared to administration of *M. vaccae* prior. (# vs *M. vaccae* prior + BBS during). D) CSC mice of Veh and BBS prior + *M. vaccae* during treatments spent significantly more time in corners than the respective SHC.

Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. For each group N=8, except CSC Veh N=7.

* for $p \leq 0.05$ vs. respective SHC group.

for $p \leq 0.05$ vs. respective BBS prior + BBS during (Fig. 21A) and M. vaccae prior + BBS during (Fig. 21C) group.

OF: open field; NO: novel object; BBS: borate-buffered saline; *M. vac*: *Mycobacterium vaccae.*

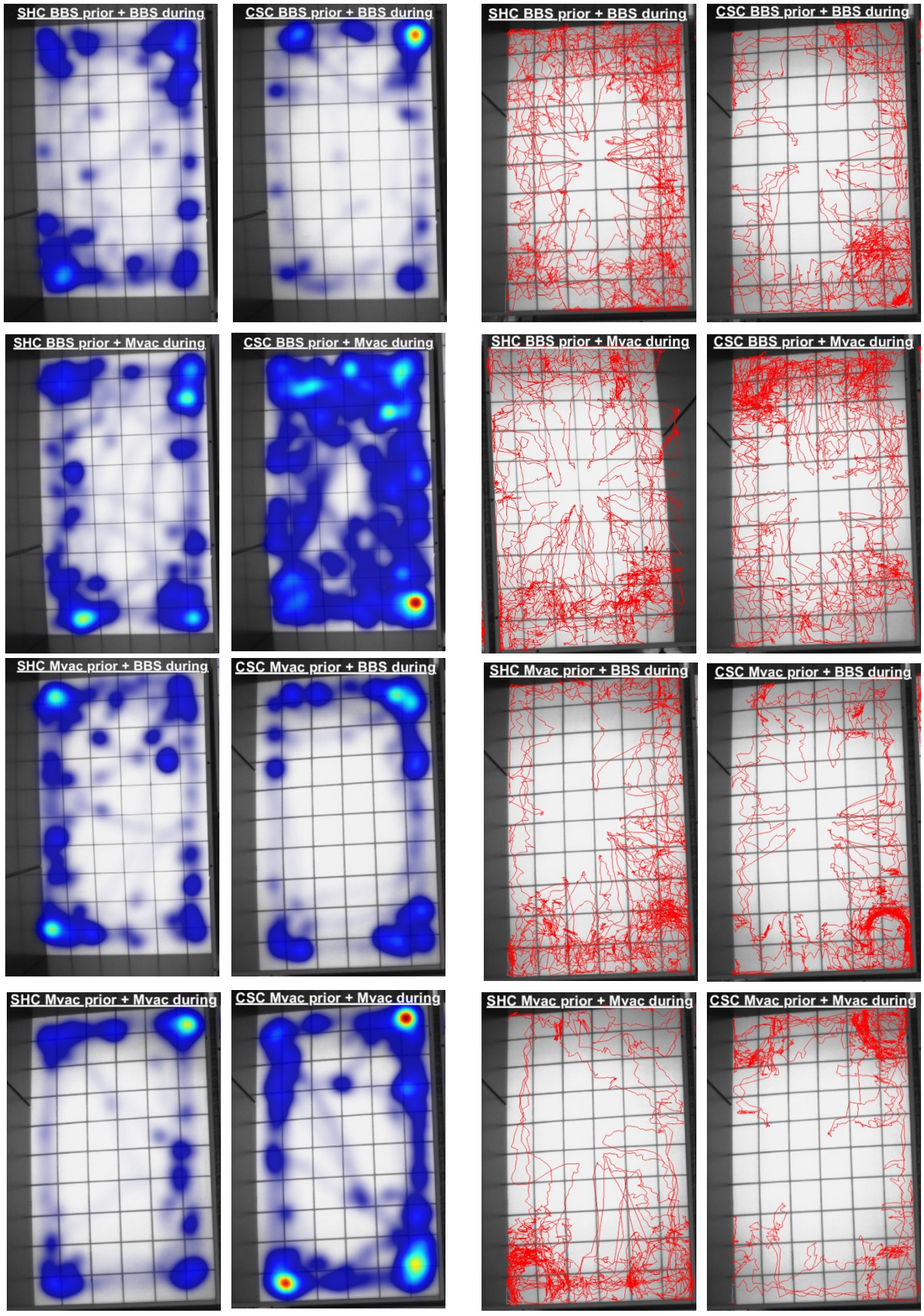

Figure 22 By way of illustration, **t**racking images displaying locomotor activity of representative subjects from each experimental group in the open field test (blue, center point tracking, left side of image) and in the novel object test (red, nose point tracking, right side of image) are shown**.**

3.3.2 Social anxiety

•SPAT

Mice were tested for social anxiety-related behavior on the SPAT on day 21 of the experimental protocol (Figure 2).

Total distance moved was significantly increased in the social vs. empty compartment in the SHC BBS prior + BBS during group (Fig. 23A; Wilcoxon; p = 0.012) and in the SHC BBS prior + *M. vaccae* during group (Fig. 23A; Wilcoxon; p = 0.012).

Time spent in direct contact was significantly increased in the social vs. empty compartment in the SHC BBS prior + BBS during group (Fig. 23B; Wilcoxon; p=0.012), in the SHC BBS prior + *M. vaccae* during group (Fig. 23B; Wilcoxon*;* p=0.050) and in the SHC *M. vaccae* prior + BBS during group (Fig. 23B; Wilcoxon; p=0.025).

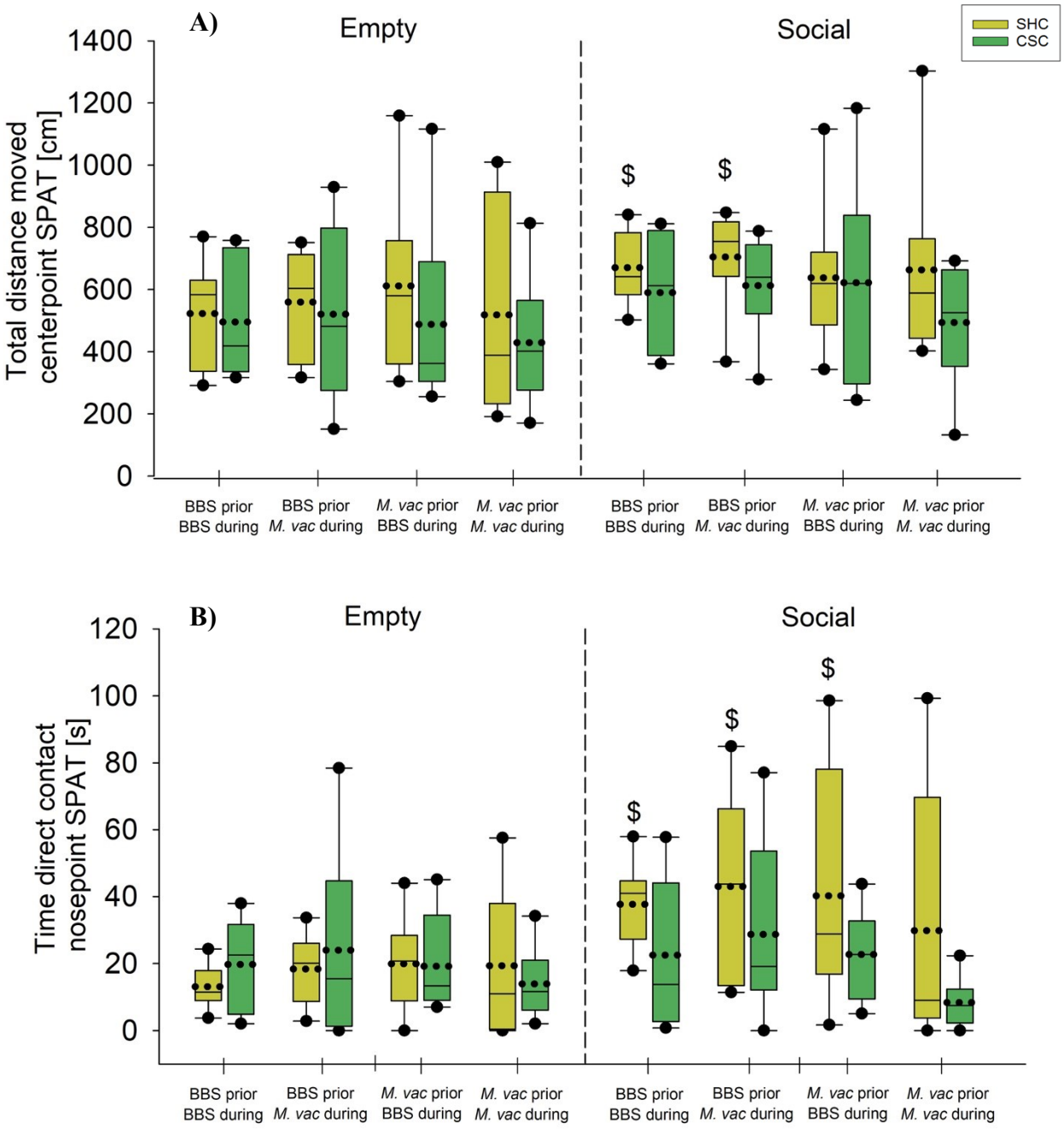

Figure 23 Effects of repeated intragastric administration of *Mycobacterium vaccae* (*M. vaccae*) on SPAT.

A) SHC Veh and BBS prior + *M. vaccae* during mice show a higher distance moved during social condition when compared to respective groups in the empty condition. B) SHC Veh, BBS prior + *M. vaccae* during and *M. vaccae* prior + BBS during mice spend more time in direct contact zone during social condition if compared to the respective group in the empty condition.

Non-parametric data are represented as boxplots. Solid line represents the median, dotted line represents the mean for each data set. Lower boxes indicate 25th percentile while upper boxes indicate 75th percentile; 10th (lower error bar), and 90th percentile (upper error bar) as well as possible outliers beyond the percentiles (indicated by closed circles) are also shown. For each group N=8, except for CSC Veh N=7.

$ for P≤0.05 vs. respective group in empty condition.

BBS: borate-buffered saline; *M. vac: Mycobacterium vaccae*.

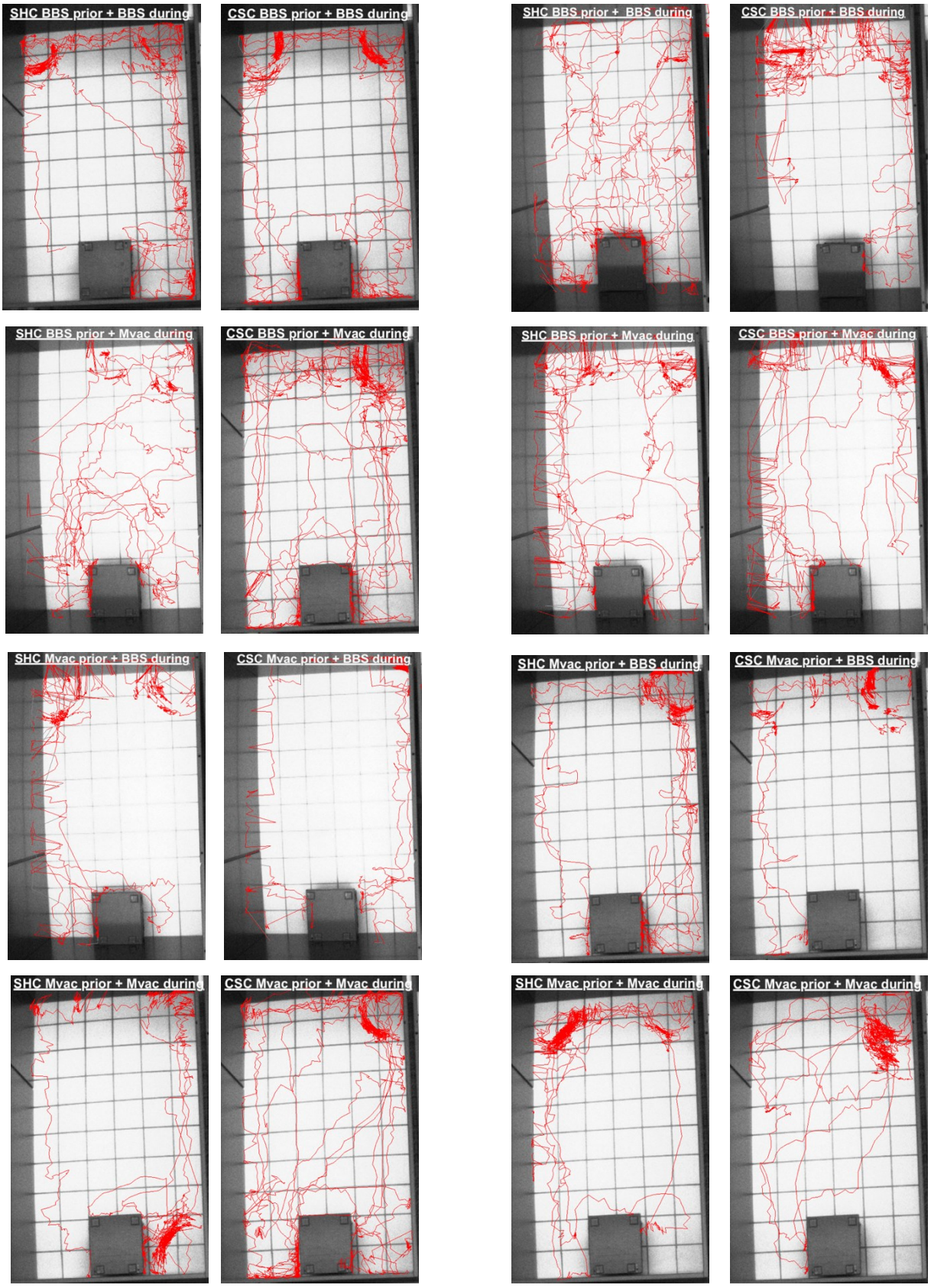

Figure 24 By way of illustration, tracking images displaying locomotor activity of representative subjects from each experimental group in the empty (red, nose point tracking, left side of image) and in the social (red, nose point tracking, right side of image) compartment of the social preference avoidance test are shown.

CHAPTER

Discussion 4

This thesis aimed to investigate the stress-protective effects of a heat-killed preparation of *M. vaccae,* administered intragastrically prior to and during chronic psychosocial stress, on physiological, immunological and behavioral parameters. To investigate the stress-protective effects of *M. vaccae*, the CSC paradigm was used. A total of eight groups of mice (SHC/CSC BBS prior + BBS during, SHC/CSC BBS prior + *M. vaccae* during, SHC/CSC *M. vaccae* prior + BBS during, SHC/CSC *M. vaccae* prior + *M. vaccae* during) were compared to assess whether the prolonged administration of *M. vaccae* both prior to- and during- CSC exposure confers protection against chronic psychosocial stress.

4.1 *M. vaccae* effects on body weight during CSC

Even if many CSC studies report a decrease in body weight of stressed mice (Reber et al., 2007; Schmidt et al., 2010; Singewald et al., 2009), others did not observe any difference compared with controls (Füchsl et al., 2014; Slattery et al., 2012; Veenema et al., 2008). In the current settings, an increased body weight gain is observed in the CSC BBS prior + *M. vaccae* during group, compared to the respective SHC group: the groups treated with *M. vaccae* prior to CSC exposure, as well as treated with *M. vaccae* prior + during and BBS prior + BBS during, did not show differences between SHC and CSC in terms of body weight gain.

4.2 Effects of *M. vaccae* on organs weight

Previous studies showed adrenal glands hypertrophy in mice following CSC exposure (Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2012). Moreover, *M. vaccae* treatment did not affect adrenal weight (Reber et al., 2016). In line with the literature, the present study showed adrenal enlargement in CSC vs. SHC mice of all groups, except the *M. vaccae* prior + BBS during group. Nevertheless, given that the combined treatment of *M. vaccae* prior + *M.*

vaccae during did not correct stress-induced adrenal enlargement, it is unlikely that the lack of effects in the *M. vaccae* prior + BBS during group could result from a protective effect of *M. vaccae.* Therefore, the lack of significance in the latter group could be due to less aggressive dominant mice in the CSC cage or to a low animal number.

As previously described, development of splenomegaly is highly dependent on bite wounds received during CSC exposure (Foertsch et al., 2017). Since all mice can be considered as equally bitten (Fig. 17), the significantly increased spleen weight observed in CSC *M. vaccae* prior + BBS during and *M. vaccae* prior + *M. vaccae* during mice is likely to indicate that *M. vaccae* has no protective effects against wounding-induced immigration of CD11b+ cells into the spleen during CSC. Moreover, the significant difference expected to be observed between SHC and CSC in BBS prior + BBS during mice is not shown. This lack of effect in the Veh group could be due to a low animal number (N=7) compared to the other N groups.

It is known from literature that GCs can induce apoptosis and inhibit immune cells proliferation (McEwen et al., 1997; Schwartzman & Cidlowski, 1994). Therefore, chronically elevated GCs may play an important role in CSC-induced thymus atrophy (Langgartner et al., 2015; Reber et al., 2007), especially considering that the thymus contains immature CD4+/CD8+ cells that are particularly sensitive to GCs, given their high expression of GC type-II receptors (Ashwell et al., 2000; Lowy, 1989).

Evidence of stress-induced thymus atrophy is also shown in this the current settings: it is present in all groups, except for the one treated with *M. vaccae* prior to and during CSC exposure, indicating a possible beneficial effect of the prolonged administration of *M. vaccae* on thymus mass development in young mice under stressful conditions.

Nevertheless, as plasma corticosterone was found higher in CSC vs. SHC *M. vaccae* prior + BBS during group only, GCs are unlikely to be the only reason to explain CSC-induced thymus

atrophy. It is known that the medullary part of the thymus is also sensitive to catecholamines, as it expresses beta adrenergic receptors at high density (Bulloch & Pomerantz, 1984; Williams et al., 1981), which are involved in cAMP-mediated apoptosis of thymocytes (Gu et al., 2000; Rey et al., 2003). Therefore, the decreased thymic mass found in our study after CSC may be a consequence of an increased sympathetic nervous system activation. Moreover, in support, CSC *M. vaccae* prior + BBS during mice have been attacked by the dominant in the cage a few minutes before they were euthanized, inducing a probable increase in GCs levels.

Accordingly, the protective effect of the combined administration of *M. vaccae* both prior to and during CSC exposure might therefore be a consequence of the action of *M. vaccae* reducing the activity of the sympathetic nervous system.

4.3 Effects of *M. vaccae* on innate and adaptive immune system

To assess the effects of CSC and *M. vaccae* administration on the innate immune system, splenocytes were isolated and stimulated *in vitro* with LPS, to induce cell proliferation. To assess the sensitivity of splenocytes to GCs, *in vitro*-stimulated splenocytes were treated with different CORT concentrations (0 μM, 5 μM). Following the stimulation, cell viability was measured. A high GCs resistance is related to low GCs sensitivity and it is indicated by higher cell viability at increasing CORT concentrations.

GCs resistance is a state of reduced sensitivity to the suppressive effects of GCs on immune cells proliferation. Since GCs are the main effectors of the HPA axis, GCs resistance has been investigated in splenic immune cells to assess whether the different treatments were effective against chronic psychosocial stress.

CSC is known to cause splenomegaly (Füchsl et al., 2014), an effect that is influenced by the number and intensity of bite wounds received (Foertsch et al., 2017). All animals used for this work had no differences regarding the bite score, so they can be considered as equally bitten.

The lack of significant differences in the cell viability of SHC vs. CSC BBS prior + BBS during mice in both basal and LPS-stimulated conditions (Figure 15) might be due to the low animal number of this settings. CSC in this treatment group has N=7, whereas all other groups have N=8. This difference in n number among the group may explain why no statistical difference could be detected between SHC and CSC in the aforementioned group. Also, it is likely the case that by increasing the animal number to an N > 8, the effect of the stimulation could also be seen in the CSC vs. SHC BBS prior + BBS during group.

An increased delta cell viability at CORT=5µM (Figure 16A) is observed in CSC BBS prior + BBS during vs. respective SHC mice, showing GCs resistance. This treatment effect can be explained by *M. vaccae* administration, which acts by stimulating immune system also in absence of stressors or pathogens.

Finally, GCs resistance is evident only in CSC BBS prior + BBS during, indicating that all the other treatments determine a protective effect (Figure 16B). Splenocyte activity under basal and LPS-stimulated conditions, as well as the delta of these two conditions, was affected by chronic psychosocial stress. Thus, *M. vaccae* is stimulating immune response for all the treatments, inducing anti-inflammatory and immunoregulatory effects independent on timepoint of administration of the bacterium.

4.4 Effect of *M. vaccae* on anxiety-related behaviors

To investigate the effects of CSC and *M. vaccae* on general and social anxiety-related behaviors, behavioral tests were performed in the current study. EZM and OF/NO test were performed to evaluate general anxiety-related behavior. Recent evidence from our group showed that the subcutaneous (s.c.) administration of *M. vaccae* prevented CSC-induced general anxiety when administered during CSC exposure (Amoroso et al., 2019). In this study, no differences between groups were observed neither in the total distance moved, nor in the percent of time spent in open arms relative to the time spent in all arms in the EZM. This is likely due to the fact that the intragastric administration procedure requires the immobilization of experimental mice and, thus, is per se stressful and anxiogenic, masking possible CSC effects on anxiety-related behavior. Support for this role of immobilization stress in anxiety-related behavior comes from data showing that acute immobilization stress is able to increase anxiety-like behavior with a time delay of about 10 days, measured as open arm avoidance in the elevated plus maze test (Mitra et al., 2005). This effect was paralleled by an increased spine density on the dendrites of basolateral amygdala neurons (BLA), which is believed to be a storage site of fearful memories (Blair et al., 201; LeDoux, 1993; Rogan et al., 1997; Schafe et al., 2001). These effects of immobilization on emotional behavior were already detected by our group in previous work where mice were injected intranasally (Amoroso et al., 2019). Total distance moved during novel object exploration was reduced in CSC vs. SHC mice in BBS prior + BBS during group. This difference is not detected when *M. vaccae* is present, independently of the time point of administration, indicating a protective effect of *M. vaccae* on general anxiety-like behavior. Moreover, CSC vs. SHC *M. vaccae* prior + BBS during and *M. vaccae* prior + *M. vaccae* during mice show decreased number of entries in contact zone with the novel object, indicating general anxiety-related behavior in these groups. Nevertheless, and contrary to our expectation, no difference in the number of entries in contact zone with the

novel object in SHC vs. CSC BBS prior + BBS during group was detected, making the effects of *M. vaccae* on general anxiety-related behavior difficult to interpret. This lack of effect in the aforementioned group may be a reflection of the current animal number.

The SPAT was used to test for social anxiety-related behavior: no effects were shown between SHC and CSC in any of the treatments. Only SHC mice showed an increase in locomotory activity when facing the social cage, compared to the empty cage. In detail, SHC BBS prior + BBS during, SHC BBS prior + *M. vaccae* during and SHC *M. vaccae* prior + BBS during showed social preference, defined as increased time spent in direct contact with the social compared to the empty cage. This was not true for the three CSC *M. vaccae*-treated groups.

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CHAPTER

Conclusions 5

As previous work from our group already investigated the stress-protective effects of *M. vaccae* administered intragastrically either prior or during exposure to CSC, the question whether a combined administration of *M. vaccae* both prior and during CSC exposure could boost its stress protective effect was still open. This work aimed at addressing this issue by evaluating the stress-protective effects of *M. vaccae* administered intragastrically both prior and during exposure to CSC. In the current study, the combined administration of *M. vaccae* only mildly corrected CSC-induced inflammatory and behavioral parameters, and a clear boosting effect of the combined treatment could only be seen as a prevention of CSC-induced thymus mass reduction in young mice under stressful conditions. Concerning anxiety-related behaviors, it was not possible to reliably detect anxiety-related behavior in CSC mice, possibly because of the immobilization procedure required during intragastric injections. To overcome this issue, our group is planning future experiment administering *M. vaccae* in palatable pellets, and hence avoiding the need to handle mice. Moreover, to achieve better results throughout i.g. administration, future studies could increase the dose of *M. vaccae* or plan to administer it daily.

Of note, the intragastric administration represents an interesting translational approach for humans, because of its non-invasive nature. In principle, humans would benefit from *M. vaccae* administration by simply self-administer the bacterium orally through pills. Moreover, CSC represent a good model for PTSD in humans (Reber et al., 2016) and *M. vaccae* seems to be a promising strategy to prevent, or at least ameliorate, the negative consequences of psychological traumata on mental and somatic health. More research is still required in order to highlight the mechanisms of action of *M. vaccae,* as well as the most effective administration method and timepoint.

CHAPTER

Acknowledgements 6

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Giulia

CHAPTER

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CHAPTER

Appendices 8

8.1 List of abbreviations

ACK	Ammonium-Chloride-Potassium
ACTH	Adrenocorticotropic hormone
BBS	Borate-buffered saline
BSA	Bovine serum albumin
CORT	Corticosterone
CRH	Corticotropin-releasing-hormone
CRP	C-reactive protein
CSC	Chronic subordinate colony
DMEM	Dulbecco's Modified Eagle's Medium
dPBS	Dulbecco's phosphate buffer solution
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPM	Elevated plus maze
EZM	Elevated zero maze
FACS	Flow cytometry
FACS buffer	dPBS + 10 % FCS + 0.1 % NaN_3 Fetal calf serum
FOXP3	Forkhead box protein 3
GC	Glucocorticoid
HBSS	Hanks balances salt solution
HPA axis	Hypothalamic-pituitary-adrenal axis
i.g.	Intragastric
i.n.	Intranasal
IBD	Inflammatory bowel disease
IFN-γ	Interferon γ
IL	Interleukin
LPS	Lipopolysaccharide
M. aurum	*Mycobacterium aurum*

M. vaccae	*Mycobacterium vaccae*
OD	Optical density
OF/NO	Open field/ novel object test
PBMC	Peripheral blood mononuclear cell
PFA	Paraformaldehyde
PSA	Polysaccharide A
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
RPMI	Roswell park memorial institute medium
RPMI+ Medium	RPMI + 10 % FCS + 100 U/ml penicillin + 100 µg/ml streptomycin + $3x10^{-5}$ M β-Mercapto- ethanol
s.c.	Subcutaneous
SHC	Single housed control
SNS	Sympathetic nervous system
SPAT	Social preference/ avoidance test
SPF	Specific pathogen-free
Supplemented RPMI-medium	RPMI + 10 % FCS + 100 U/ml penicillin + 100 µg/ml streptomycin
TGF-β	Transforming growth factor β
Th	T helper cell
TNF-α	Tumor necrosis factor α
Treg	Regulatory T-cell
Veh	Vehicle

8.2 List of materials

96-well plate	Falcon, USA
Anti-CD25	Invitrogen, Thermo Fischer Scientific, USA
Anti-CD28	Thermo Scientific, Czech Republic

Anti-CD3	Thermo Scientific, Czech Republic
Anti-CD4	Invitrogen, Thermo Fischer Scientific, USA
Anti-CD62L	Invitrogen, Thermo Fischer Scientific, USA
Anti-FOXP3	Invitrogen, Thermo Fischer Scientific, USA
Automated cell counter	TC-20, BioRad Laboratories, München, Germany
BBS	Immodulon, London, United Kingdom
C57BL/6N mice	Charles River, Sulzfeld, Germany
CD1 mice	Charles River, Sulzfeld, Germany
Cell strainer (70µm)	Corning, USA
Centrifugation tube (50 ml)	Falcon, USA
CORT	Sigma-Aldrich, United Kingdom
CORT ELISA	IBL international, Hamburg, Germany
DMEM	Gibco life Technologies, United Kingdom
dPBS	Gibco life Technologies, United Kingdom
EDTA-tubes	Sarstedt AG & Co, Germany
ELISA plate reader	Fluostar Optima, BMG Labtech, Offenburg, Germany
FCS	Gibco life Technologies, United Kingdom
HBSS	Sigma-Aldrich, United Kingdom
IFN-γ ELISA	R&D Systems, Minneapolis, USA
LPS	Sigma-Aldrich, United Kingdom
LSRII flow cytometer	BD, Biosciences, Heidelberg, Germany
MTS colorimetric assay	CellTiter 96 Aqueous One Solution Assay, Promega, Madison, WI
M. vaccae	Immodulon, London, United Kingdom
RPMI	Sigma-Aldrich, United Kingdom