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The Effect of *M. vaccae* Preimmunization on Anxiety- and Panic-like Behaviors

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Abstract:

The coevolution of microorganisms and their hosts have resulted in the formation of symbiotic relationships between both organisms. These formed associations have in turn benefited larger animals by enhancing their adaption towards external environments and protecting against pathogens. Recent studies have further highlighted the importance of microbial interactions by analyzing their role in proper immune regulation and their effect on behavior and health. Faulty immunoregulatory circuits have been implicated in the rapid rise of chronic inflammatory diseases and considered a risk factor for psychiatric diseases. Although studies have begun to understand the link between microbes and stress-related behaviors, few studies have focused on the potential influence of environmental bacteria on anxiety related disorders. In this study we show that rats that were subcutaneously immunized with heat-killed *M. vaccae*, a non-pathogenic environmental bacterium, expressed reduced levels of anxiety-related behaviors when tested in the elevated T-maze. As compared to the control group, *M. vaccae*-treated rats expressed significantly lower latencies when measuring inhibitory avoidance, a behavior related to generalized-anxiety. During the escape task, a behavior associated with panic behavior, *M. vaccae* treated rats had larger latencies to leave the open arm, but the differences between groups were not significant. The results of our studies suggest a beneficial effect of heat-killed *M. vaccae* on anxiety-related behaviors in the elevated T-maze. Our study supports the on-going hypothesis regarding the beneficial role of immunoregulatory environmental microbes in control of emotional behavior and emotional states.

1. Introduction

1.1 Gut Microbiome

Microorganisms are one of the oldest and most abundant groups of living organisms on earth. Being ubiquitous in nature and one of the first forms of life, bacteria have coevolved alongside other much more complex, multicellular organisms for millions of years (Rook, 2010). The inevitable constant interactions and shared evolution between macro- and micro-organisms eventually combined to form symbiotic associations that established dependency between both groups (Rook, 2010). A major site of bacterial exposure and inhabitation in mammals is the gastrointestinal (GI) tract. Bacteria residing within the GIT have, through time, become integral components in their host's immune regulation (Rook, 2012). In the last several decades, research studies have begun highlighting the importance of the mutual dependency between bacterial communities and their host (McFall- Ngai et al., 2013; Gilbert et al., 2012). Through a myriad of studies it has become clear that the symbiotic relationships, formed by microorganisms and their hosts, have aided animals with an enhanced adaptation to their daily environment as well as providing protective benefits from malignant pathogens (Bach, 2002; Rook, 2012; Rook and Lowry, 2008).

Furthermore, recent studies in rodents (Bercik et al., 2011) and human subjects (Knowles et al., 2008) have added to our understanding by looking at ways in which interactions with the gut microbiome can influence behaviors. For example, Bravo et al. (2012) showed that mice treated with probiotics had a better performance in the forced swim test and the elevated plus-maze test, indicating reduced depressive- and anxiety-like behaviors, respectively. On the other hand, additional studies have analyzed anxiety-like behaviors after experimentally disrupting the gut microbiome through antibiotics or bacterial pathogens. Gaykema and colleagues (2004) conducted an experiment where mice expressed increased anxiety-like behavior and activation in brainstem regions related to anxiety after a cecal infection with *Campylobacter jejuni*, a gram-negative bacterial pathogen.

The physiological and behavioral outcomes resulting from such symbiotic relationships rely on the ability of microbial communities, within the GIT, to

communicate with the brain via the known microbiota-gut-brain axis (Cryan and O'Mahony, 2011). This bidirectional signaling between the gut microbiota and the central nervous system (CNS) is particularly important for the autoregulation of physiological systems such as the endocrine, immune, and both branches of the autonomic nervous system (Grenham et al., 2011). The previously mentioned investigations are only some of many studies elucidating the importance of the gut microbial state in the regulation of an animal's physiology, functioning of its CNS and influence in certain behaviors. Despite this evidence, formulating the composition parameters for a healthy gut microbiota, both quantitatively and qualitatively, is a challenging and on-going process. It does, however, introduce the idea of possibly being able to seek new therapeutic and preventative avenues for a range of diseases and disorders.

1.2 The "Old Friends" Hypothesis

Microbial communities that have colonized the human gut do not appear to be the only organisms influencing physiological regulation. Our exposure and ability to tolerate environmental, non-pathogenic microbes and helminths (parasitic worms) has also gained a lot of attention as an important factor in maintaining our health and wellbeing. The idea that environmental microbes have the capacity to influence our immune system and in turn our health was first proposed in the late 1980s and named the 'hygiene' hypothesis (Rook and Brunet, 2005). Since then, the original idea has been amplified and presented as the "Old Friends" hypothesis by Rook and colleagues. The goal of the "Old Friends" hypothesis is to provide a link between rising levels of chronic inflammatory diseases in developed countries, modern living conditions and reduced exposure to immunoregulatory microbes. Chronic inflammatory diseases are characterized by a prolonged or hyper activated immune response and can encompass autoimmune diseases, allergies and inflammatory bowel disease (Rook et al., 2013; Heap et al. 2009).

The "Old Friends" hypothesis proposes that the rapid rise in chronic inflammatory diseases, which are particularly prevalent in first world societies, is partially due to a diminished exposure to non-pathogenic, immunoregulatory "Old Friends" (Rook and

Lowry, 2008; Rook et al., 2013). In other words, the coexistence of the human body with harmless bacteria and helminths, for the past thousands of years, has served our bodies by developing an 'educated' immune system able to control and avoid inappropriate inflammatory responses. Over time, however, our interactions with the wide range of immunoregulatory agents have decreased due to changes in our lifestyle including: modern diets, living conditions, and use of antibiotics (Rook et al., 2013). The interference with long established relationships has in turn created disruptions in immunoregulatory circuits, and affected the immune system's ability to know when an inflammatory response is detrimental (Rook et al., 2012; Matthews and Jenks, 2013). Faulty immunoregulatory mechanisms have been implicated in rising levels of chronic inflammatory diseases, but also attributed to some stress-related psychiatric disorders (Rook and Lowry, 2008; Rook et al., 2012, 2013). Interestingly, the treatment with certain species of harmless bacteria such as *Mycobacterium vaccae* (*M. vaccae*) can induce an anti-inflammatory response through the activation of regulatory T cell (T_{reg}) lymphocytes in mice (Zuany-Amorim et al., 2002). *M. vaccae*, an environmental bacteria belonging to the "old friends" category, is receiving increasing attention by research teams investigating psychiatric diseases where inflammation is considered a risk factor.

1.3 *Mycobacterium vaccae* (*M. vaccae*)

M. vaccae is a non-pathogenic, saprophytic bacterium that has been used in multiple studies to investigate whether exposure to ambient saprophytes can improve the condition of diseases associated with elevated inflammatory activity or the emotional behavior related to them (Matthews and Jenks, 2013; Camporota et al., 2003; Wang and Rook, 2003). Among these studies is O'Brien et al. (2004), which demonstrated improved emotional state and general cognitive function after terminal lung cancer patients were administered *M. vaccae*.

One of the most important monoaminergic neuromodulators implicated in mood, arousal and cognitive processes is serotonin (Cools et al., 2008; Van der Veen et al., 2007). The serotonergic system is involved in the etiology and pathophysiology of anxiety disorders. A preclinical study conducted by Dr. Lowry and coworkers (2007) investigated how the preimmunization with heat-killed *M. vaccae* can affect the dorsal

raphe nucleus. The dorsal raphe nucleus (DR) is a major source of serotonergic projections to limbic structures involved in the modulation of fear and anxiety (Azmitia and Segal, 1978; Vertes, 1991). Lowry et al. (2007) revealed that a distinct subset of serotonergic neurons in the interfascicular region of the dorsal raphe nucleus (DRI) was activated and that levels of serotonin metabolism in the medial prefrontal cortex (mPFC) were increased. In this same study, reduced stress-related behaviors in the forced swim test were also observed, analogous to antidepressant treatment effect (Petit-Demouliere et al., 2005). Despite these findings, the direct relationship between anxiety-like behaviors and *M. vaccae* treatment remains unclear and insufficiently studied. To better understand this interaction, we investigated if the preimmunization of rats with heat-killed *M. vaccae* is able to inhibit the expression of anxiety-like behaviors in the elevated T-maze. This behavioral test is derived from the elevated plus-maze and allows for the measurement, in the same rat, of two distinct anxiety-like behaviors related to different types of anxiety disorders (Zangrossi and Graeff, 2014). Generalized anxiety- and panic-related defensive responses can be evaluated by the measurement of inhibitory avoidance and escape behaviors, respectively (Zangrossi and Graeff, 2014).

2. General Materials and Methods

2.1 Animals

Adolescent male Wistar rats (HSD-WI, Harlan Laboratories, Indianapolis, IN, USA; N=24) arrived from the vendor weighing approximately 150 g. Upon arrival, rats were pair-housed in standard polycarbonate breeding cages (Makrolon type II; 265mm L x 205 mm W x 140 mm H); food (Cat. No. 2018, Teklad I 8640 22/5 Rodent Diet, Harlan, Madison, WI, USA) and tap water were available *ad libitum* under controlled, constant laboratory conditions (12:12 h dark/light cycle with lights on starting at 07:00 h; room temperature 22 °C; air humidity 20%). All experimental procedures were conducted during the light phase and in accordance with the *Guide for the Care and Use of Laboratory Animals*, Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, 2011) and were approved by the

University of Colorado Boulder Institutional Animal Care and Use Committee
(Addendum 5/ 2134-14MAY2018).

2.2 *M. vaccae*

Heat-killed *M. vaccae* (NCTC # 11659) used for this experiment was obtained from Immodulon Therapeutics, London, UK, manufactured by Eden Biodesign (Liverpool, UK) and shipped by BioElpida (Lyon, France).

2.3 Apparatus

The elevated T-maze was made of wood and had three arms of equal dimensions (50 x 12 cm). One arm, enclosed by 40 cm-high walls, was perpendicular to two opposed open arms. To avoid falls, the open arms are surrounded by a 1 cm-high (polymethyl methacrylate; Plexiglas®) rim. The described platform is 50 cm. above the ground (Fig. 1).



Fig. 1. Picture of the elevated T-maze apparatus used to measure inhibitory avoidance and escape behaviors in this experiment.

2.4 Experimental Design

As Figure 2 illustrates, rats arrived on day -28 (days prior to initiation of testing on the elevated T-maze are denoted as negative). Upon arrival, rats were housed in the University of Colorado Boulder Muenzinger's animal housing unit and given a one-week acclimation period. After the seven-day acclimation period, rats were randomly pre-immunized subcutaneously (s.c.) with either heat-killed *M. vaccae* antigen (n=12; 0.1 mg/100 µl) or with sterile borate buffered saline (BBS) vehicle (n=12; 100 µl) on days -21, -14, and -7. In an effort to eliminate unconscious bias, the experimenter was blinded to treatment groups. On days -3 and -2, rats were subjected to five-minute sessions of handling by the experimenter. These consecutive handling sessions are designed to get rats accustomed to being handled by the experimenter and reduce unintended stressors on test day. On day -1, all rats were pre-exposed and confined to one of the open arms in the elevated T-maze for thirty minutes. A wooden barrier mounted on the border between the maze central area and the proximal end of the open arm isolated this arm from the rest of the maze. It has been shown that this pre-exposure to the open arm renders the escape task more sensitive to the effects of anti-panic drugs, because it shortens the latencies of withdrawal from the open arm during the test (Poltronieri et al., 2003; Teixeira et al., 2000). Twenty-four hours later, on day 0, rats were tested in the elevated T-maze.

Three days after being tested on the elevated T-maze, rats were subjected to stereotaxic surgeries and behavioral tests for other purposes. They were ultimately euthanized with anesthetic overdose (Fatal Plus®; Vortech Pharmaceuticals LTD.; Dearborn, Michigan, USA, 2 mg/kg).

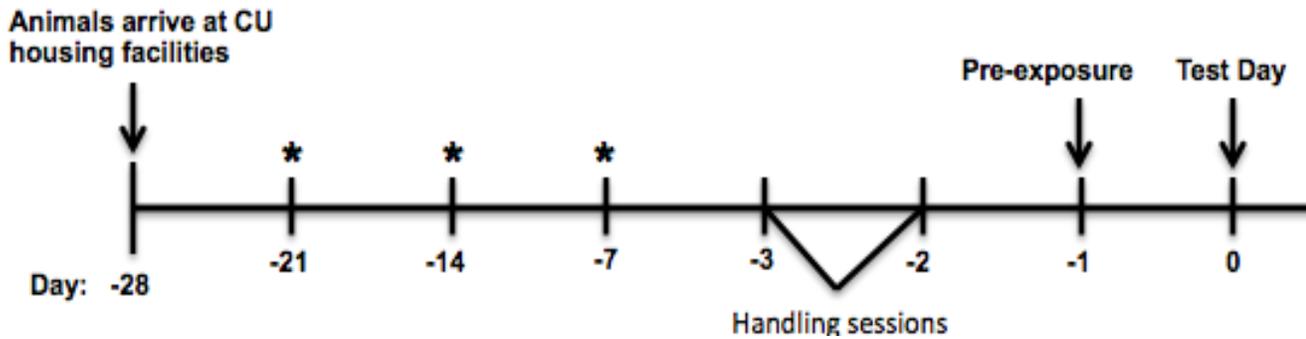


Fig. 2. Schematic illustration of the experimental timeline. The one-week acclimation period begins on day -28. * indicates days on which rats were immunized with either heat-killed *M. vaccae* antigens or BBS. Handling sessions were on days -3 and -2 while pre-exposure on the elevated T-maze open arms occurred on day -1. On test day (Day 0), rats were first tested on the enclosed arm then the open arm of the elevated T-maze.

2.5 Experimental Procedures

As previously mentioned, the elevated T-maze test portion of the experiment was conducted on day 0 and under 60 lux of light intensity, measured at the enclosed arm. The experiment began by the measurement of inhibitory avoidance, which has been related to generalized anxiety (Zangrossi and Graeff, 1997). In order to evaluate this anxiety-like behavior, each animal was placed at the distal end of the enclosed arm, facing the intersection of all arms, and the amount of time it took the rat to withdraw from this arm with all four paws (referred as latency and measured in seconds) was recorded. Three distinct trials were recorded separated by 30-second inter-trial intervals. During the 30-second inter-trial intervals, rats were placed in an experimental cage. The first recording of the inhibitory avoidance for each rat constituted its baseline latency measurement while the two subsequent trials were considered avoidance #1 and avoidance #2, respectively. Following the completion of avoidance #2 and a 30-second interval in the experimental cage, each rat was placed at the end of the respective open arm they were pre-exposed to on the previous day and facing the middle of the apparatus. The latency to withdraw from the open arm with all four paws by each rat was considered the end of the escape task. A total of three escape trials separated by 30-second inter-trial intervals were recorded. As before, the 30-second inter-trial intervals were spent in the experimental cage. The latter half of the elevated T-maze analyzes unconditioned fear, which has been associated with panic disorders

(Zangrossi and Graeff, 1997). A cut-off time of 300 seconds was established for the avoidance and escape latencies.

3. Statistics

All statistical comparisons were performed in Statistical Package for the Social Sciences (SPSS) version 22.0 (SPSS, Chicago, IL, USA). Prior to statistical analysis, outliers were identified in the elevated T-maze data set using the Grubbs method for single extreme outliers (Grubbs, 1969). Two inhibitory avoidance data points were removed; one being a baseline measurement from a control rat and the other an avoidance 1 measurement from an *M. vaccae*-treated rat. A single outlier was identified from an *M. vaccae*-treated rat in escape 1. The second and third escape trials had two outliers each; one outlier per treatment group. Combined, a total of seven outliers were removed from the overall data set. After removing all identified extreme outliers, mean log-transformed inhibitory avoidance latencies and escape latencies of the two treatment groups were analyzed separately using a linear mixed model (LMM) with repeated measures (trial). Treatment groups, trials, and interactions between trials and treatment were used as fixed factors. By examining the -2 Restricted Log Likelihood value (value of criteria function used to measure goodness of fit), an unstructured covariance setting was selected. A linear mixed model analysis was preferred over a repeated measures ANOVA because of its ability to more effectively estimate model parameters in unbalanced experimental designs, account for missing data point arising from Grubb's outlier calculations, and higher versatility model fitting through feature of covariance structures (Krueger and Tian, 2004; Cnaan et al., 1997). When appropriate, between-group comparisons within each trial were made using Fisher's least significant difference test (LSD) with a significance threshold of $p < 0.05$.

4. Results

M. vaccae treatment impaired inhibitory avoidance behavior expression in the elevated T-maze, indicating an anxiolytic-like effect. The results obtained for inhibitory avoidance latencies are shown in Table 1 and plotted in Figure 3. An overall increase in avoidance performance was seen in both groups. LMM analysis indicated a significant

effect of trials [$F_{(2, 22.2)}=18.25, p < 0.001$], *M. vaccae* treatment [$F_{(1, 23.1)}=5.11, p=0.034$], but not *M. vaccae* treatment x trial interaction effect [$F_{(2, 22.2)}=1.33, p=0.286$] on avoidance latencies. The differences between treatment groups in avoidance 1 and 2 were statistically significant and are indicated by the Fisher's LSD post-hoc test, $p=0.026$ and $p=0.012$, respectively.

As Table 1 and Figure 4 illustrate, *M. vaccae* treatment did not affect the escape behavior expression measure in the elevated T-maze. Contrary to inhibitory avoidance, the LMM analysis indicated no significant effect of treatment [$F_{(1, 22.4)}=0.26, p=0.617$], trials [$F_{(2, 22.2)}=1.42, p=0.262$], or treatment x trial interaction effect [$F_{(2,22.2)}=1.87, p=0.178$] on escape latencies (Table 2).

Table 1. Means \pm SEM of latencies in repeated trials of inhibitory avoidance and escape behaviors measured in the elevated T-maze.

Behavior	Treatment Group & Trial	Latency (s)
Inhibitory Avoidance	Vehicle	
	<i>Baseline</i>	14.62 \pm 3.61
	<i>Avoidance 1</i>	69.48 \pm 29.47
	<i>Avoidance 2</i>	152.64 \pm 34.97
	<i>M. vaccae</i>	
	<i>Baseline</i>	10.22 \pm 2.12
	<i>Avoidance 1</i>	13.54 \pm 3.91*
	<i>Avoidance 2</i>	73.70 \pm 31.49 *
Escape Task	Vehicle	
	<i>Escape 1</i>	7.47 \pm 1.00
	<i>Escape 2</i>	10.61 \pm 2.94
	<i>Escape 3</i>	9.50 \pm 2.97
	<i>M. vaccae</i>	
	<i>Escape 1</i>	7.51 \pm 1.02
	<i>Escape 2</i>	10.75 \pm 3.65
	<i>Escape 3</i>	14.69 \pm 4.59

* $p < 0.05$ compared to vehicle treatment

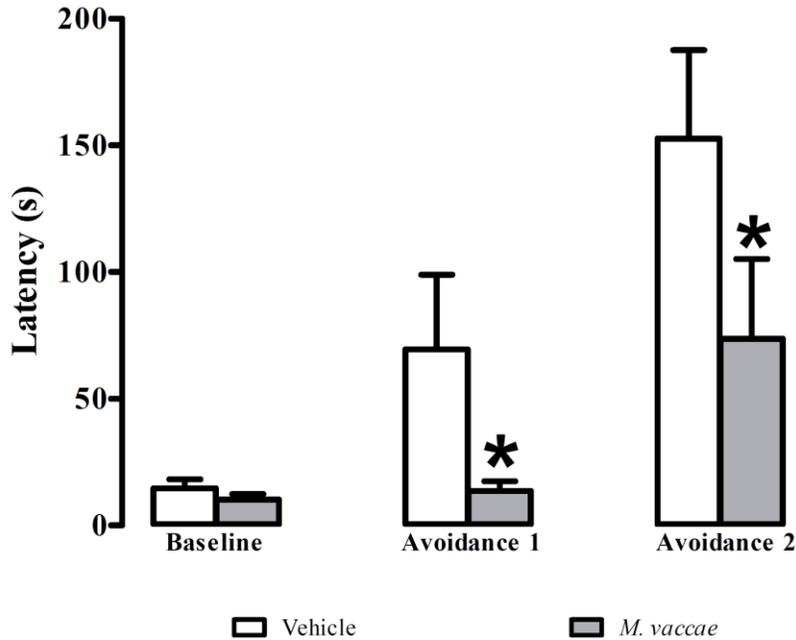


Fig. 3. Effects (means \pm SEM) of s.c injected heat-killed *M. vaccae* (n=11-12) on inhibitory avoidance latencies measured in the elevated T-maze. * indicates statistical significance, as compared to BBS vehicle group (n=11-12), at $p < 0.05$.

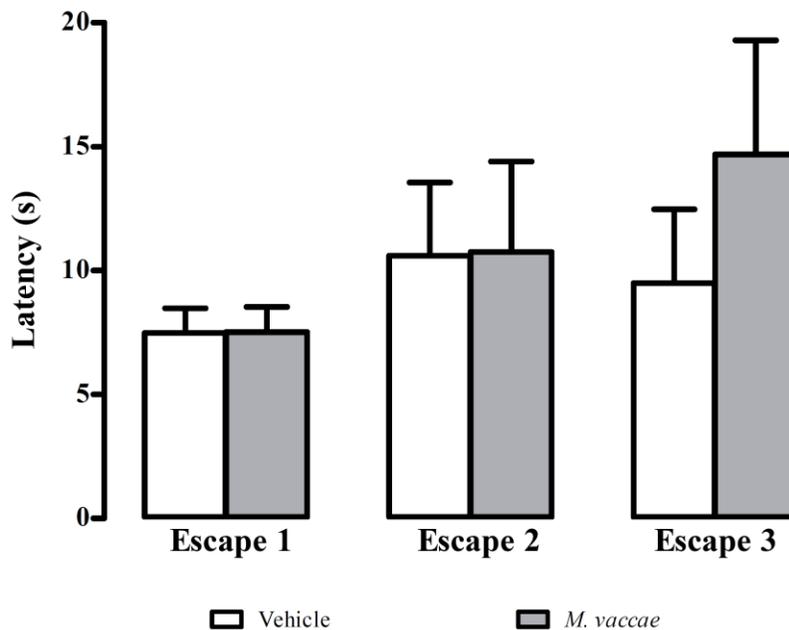


Fig. 4. Effects (means \pm SEM) of s.c injected heat-killed *M. vaccae* (n=11) on escape behavior latencies measured in the elevated T-maze. No statistical significance, as compared to BBS vehicle group (n=11-12) was found.

Discussion

The findings in this experiment suggest that s.c. immunizations of rats with heat-killed *M. vaccae* can have protective effects by inhibiting generalized anxiety-like behavior (avoidance behavior) during the elevated T-maze test. Avoidance behavior was significantly different in the experiment, but was particularly noticeable in trials following baseline measurements. Rats in both treatment groups began with a similar baseline measurement. After baseline, *M. vaccae*-treated rats presented an impaired inhibitory avoidance acquisition compared to the group treated with vehicle. In other words, they presented lower latencies to leave the enclosed arm, indicating an anxiolytic-like effect in our animal model.

Unlike the inhibitory avoidance aspect of the test, *M. vaccae* did not significantly affect the escape task, associated with panic-like behavior, in the elevated T-maze. Both treatment groups expressed similar escape latencies.

Previous studies, as mentioned before, have shown that *M. vaccae* treatment induces an anti-inflammatory effect and this can reflect in an inhibition of the stress-induce behavior (Lowry et al., unpublished). Here *M. vaccae* treatment could possibly act by reducing the inflammatory condition induced by the exposure to the aversive components of the elevated T-maze. The control group clearly avoided the open arms and spent a higher amount of time in the enclosed arm before deciding to step into the open arms. *M. vaccae* rats left the enclosed arm faster, showing decreased avoidance of the open arms.

M. vaccae's effect in diminishing the expression in one, out of two, anxiety-related behaviors tested by the elevated T-maze, further supports the current hypothesis regarding the capacity of microorganisms to positively influence chronic inflammatory diseases, like anxiety disorders, and behaviors associated with them. The composition of the gut microbiome is an important aspect to consider when looking at microbial-host interactions, but also important are the numerous relationships formed with environmental bacteria. Environmental saprophytes found in mud, soil and untreated water are examples of non-pathogenic microbes that we've been in direct contact throughout human evolution, but now have reduced exposure to (Rook et al., 2013). Although these microbes do not necessarily colonize the GIT, they've become

essential to the development of our immune system due to the great abundance and frequency in which they have interacted with our bodies for the past thousands of years. Revisiting the findings of Lowry et al. (2007) who demonstrated that peripheral exposure to heat-killed *M. vaccae* activates a subset of serotonergic neurons in the DRI and reduces stress-related behaviors, we can reason that *M. vaccae*'s effect on behavior is highly dependent on its proper interaction with both the immune system and the activation of the serotonergic pathway. Even though our study did not examine the specifics of the potential pathway, there are some who have hypothesized the route of interaction to be mediated by cytokines via afferent pathways including some found in the spinal cord (Maes et al., 1998; Maier et al., 1998).

In this experiment, *M. vaccae* treatment did not completely prevent a rise in avoidance behavior. Avoidance 2 showed an increase in *M. vaccae*-treated rats, but this increase was significantly lower and delayed than that in the control group. If our hypothesis is that *M. vaccae*'s protective effects are mediated via the activation of anti-inflammatory regulatory mechanisms, it makes sense to further validate this hypothesis and explore the possibility of disrupting such protective effects by hindering the activation of those same pathways. Current plans by our group include building on these findings and investigating whether *M. vaccae*'s protective effects can be altered by blocking anti-inflammatory cytokines such as IL-10 or transforming growth factor beta, which are responsible for initiating anti-inflammatory responses.

The elevated T-maze is designed to separate anxiety- and panic- like behaviors within one subject animal, but has also been a model prone to variability. Matthews and Jenks (2013) studied the effects of *M. vaccae* on anxiety-like behaviors and memory using a complex maze. Their finding also concluded a significant effect of *M. vaccae* on anxiety-related behaviors. In addition, the Lowry group is also analyzing the effect of *M. vaccae* on anxiolytic behavior by using the Vogel test, an experimental tool able to measure conflict anxiety. The possibility of using *M. vaccae* or other "old friends" in the prevention or treatment of anxiety disorders begins to appear as an exciting and novel possibility. Taking into account that the development of a person's immune system is highly dependent on the formation of the microbiome and interaction with environmental bacteria early on in life (Grenham et al., 2011), it will be important for future studies to

investigate weather exposure to these “old friends” has longer lasting effects if administered early on in life.

There is no question that microbial-human interactions affecting health and disease will continue to occupy a special niche within the research community. The multidisciplinary approaches that have brought us thus far will be especially important in meeting the exiting challenges that lie ahead. The likelihood is that we have only seen the beginning of many new advances in this rapidly expanding area of research.

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