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Mycobacterium vaccae immunization protects aged rats from surgery-elicited neuroinflammation and cognitive dysfunction

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Abstract

Aging is a major risk factor for developing postoperative cognitive dysfunction (POCD). Neuroinflammatory processes, which can play a causal role in the etiology of POCD, are potentiated or primed as a function of aging. Here we explored whether exposure to a microorganism with immunoregulatory and anti-inflammatory properties, *Mycobacterium vaccae* NCTC11659 (*M. vaccae*), could ameliorate age-associated neuroinflammatory priming. Aged (24 mos) and adult (3 mos) male F344XBN rats were immunized with heat-killed *M. vaccae* (3 injections, once per week) prior to undergoing a laparotomy or anesthesia control procedure. Aged, but not young rats, showed post-operative learning/memory deficits in a fear conditioning paradigm. Importantly, *M. vaccae* immunization protected aged rats from these surgery-induced cognitive impairments. *M. vaccae* immunization also shifted the aged pro-inflammatory hippocampal microenvironment towards an anti-inflammatory phenotype. Furthermore, *M. vaccae* immunization reduced age-related hyperinflammatory responses in isolated hippocampal microglia. Overall, our novel data suggest that *M. vaccae* can induce an anti-inflammatory milieu in the aged brain and thus mitigate the neuroinflammatory and cognitive impairments induced by surgery.

Keywords

aging; microglia; neuroimmune; POCD; T cells; surgical stress

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1. Introduction

Life expectancy continues to rise worldwide. Although humans are living longer, they are not necessarily healthier than before: older individuals (> 60 years) account for approximately one-quarter of the global burden of disease (Prince et al., 2015). Mental, behavioral, and neurological disorders have increased substantially in the elderly population in recent years (Prince et al., 2015). Indeed, aging is a significant risk factor for cognitive decline in response to hospitalization and surgery (Wilson et al., 2012), and age is the most salient predictor for the development of post-operative cognitive dysfunction (POCD) (Monk et al., 2008; Steinmetz et al., 2009). POCD is characterized by impairments in memory, concentration, and information processing following surgery and these impairments significantly impact quality of life and increase the risk of disability and mortality (Steinmetz et al., 2009).

Amplified neuroinflammatory responses to challenge occur with normal aging and likely contribute to POCD vulnerability. In humans, inflammation is positively associated with the development of POCD (Burkhart et al., 2010; Hudetz et al., 2011). Factors that potentiate neuroinflammatory responses, such as opioids (e.g. fentanyl) (Watkins et al., 2009), increase the risk for developing delirium and POCD (Burkhart et al., 2010) whereas anti-inflammatory strategies (e.g. ketamine) reduce the risk for the development of POCD (Hudetz et al., 2009). There is also substantial evidence from animal models that age-related changes in neuroinflammatory dynamics are causal factors in the development of POCD. Peripheral immune stimuli (e.g., infection or surgery) induce potentiated neuroinflammatory and behavioral changes in aged mice and rats (Barrientos et al., 2012b; Cao et al., 2010; Chen et al., 2008; Fonken et al., 2016; Godbout et al., 2005; Rosczyk et al., 2008; Terrando et al., 2010). For example, whereas adult (3 mos) rats rapidly recover cognitive function after a laparotomy procedure, aged (24 mos) rats exhibit prolonged post-surgery cognitive deficits (4+ days) (Barrientos et al., 2012b). Elevations in hippocampal interleukin (IL)-1β, a cytokine that is considered a master regulator of neuroinflammation (Basu et al., 2004), likely mediate these cognitive impairments following surgery. Several lines of evidence demonstrate that aged rodents exhibit protracted elevations in IL-1β post-surgery and that blocking IL-1β signaling with pharmacological or transgenic approaches can prevent surgery-induced cognitive deficits (Barrientos et al., 2012b; Cibelli et al., 2010). Indeed, IL-1β, as well as other pro-inflammatory mediators, negatively impact neural mechanisms (e.g., long-term potentiation (LTP)) that are involved in memory formation (Lynch, 2010).

Importantly, aged but not senescent animals do not necessarily exhibit declines in cognition or increased pro-inflammatory cytokines under steady-state conditions (Barrientos et al., 2006). Rather, in aged animals, the neuroimmune response to immune challenge (e.g., surgery or infection) is potentiated; a phenomenon termed “priming” (Cox et al., 2012; Fonken et al., 2016). Age-associated inflammatory priming also occurs at the cellular level, in that microglia show potentiated responses to immune stimuli *ex vivo* (Fonken et al., 2016; Frank et al., 2010). Primed inflammatory responses may develop with aging due to a combination of exogenous (environmental pollutants, pathogens, injury) and endogenous (changes in glucocorticoids, accumulation of danger signals, etc.) factors that accumulate over the lifespan (reviewed in (Fonken et al., 2018b).

Overly sterile conditions are one unique modern environmental divergence that may modulate the immune system. The hygiene or “old friends” hypothesis posits that a lack of exposure to micro- and macro-organisms that have evolved over time to program the immune system during development may cause it to become dysregulated (Rook, 2013). This hypothesis does not suggest that exposure to infections or outright pathogens is beneficial, but rather, that lack of exposure to environmental and symbiotic organisms that engage the immune system can be detrimental to immune function. This modern decrease in the occurrence of immune stimulation during development, relative to the microbiome with which the species evolved, applies both to humans and laboratory rodents. Mounting evidence suggests that the reintroduction of immunoregulatory micro-and macro-organisms in an overly “clean” environment can quell immune sensitization (reviewed in (Lowry et al., 2016)). For example, *Mycobacterium vaccae* (*M. vaccae*), a fast-growing and widely distributed species of saprophytic bacteria found in soil (Rook et al., 2004), can modify immune responses in both humans and rodents (Groschel et al., 2014). Immunizing adult rats with a heat-killed preparation of *M. vaccae* can protect against stress-elicited, primed, hyperactive immune responses and accompanying stress-induced behavioral impairments (Reber et al, 2016; Frank et al, 2018). Of note, heat-killed bacteria have the same peripheral anti-inflammatory impact as do live bacteria (Laudanno et al., 2006). Importantly, aged animals that are underexposed to “old friends” may be particularly vulnerable to impaired immunoregulation (Rook et al., 2013). Thus, here we hypothesized that treating aged rats with *M. vaccae* prior to undergoing a surgical procedure (i.e., laparotomy), which served as a peripheral immune challenge, would protect against primed neuroinflammatory responses and memory deficits. In agreement with this hypothesis, our results suggest that *M. vaccae* pre-immunization can inhibit the neuroinflammatory and behavioral changes induced by laparotomy in aged rats. Moreover, the protective effects of *M. vaccae* were associated with increased hippocampal expression of markers for anti-inflammatory microglia and regulatory T cells. These findings are of particular significance since *M. vaccae* NCTC 11659 can be given to humans (Groschel et al., 2014).

2. Methods

2.1. Animals

Adult (3 mos) and aged (24 mos) male F344XBN F1 rats were used in these experiments. Rats of this age and strain were selected to study healthy, non-neurodegenerative aging (these rats live for > 30 mos)(Barrientos et al., 2006). Aged rats that had overt health issues such as tumors were excluded from experiments. Rats were received from NIA and pair-housed (52 cm L × 30 cm W × 21 cm H) with an age-matched conspecific. Food and water were available *ad libitum* and rats were maintained at an ambient temperature of 22±2°C on a 12:12 light cycle with lights on at 0700. Upon arrival, rats were acclimated for 7+ days prior to any experimental manipulation. All experimental procedures were conducted in accordance with ARRIVE guidelines and were approved by the University of Colorado Boulder Institutional Animal Care and Use Committee. Efforts were made to minimize animal use and discomfort.

2.2. Experimental design

2.2.1. Experiment 1: Does M. vaccae immunization protect aged rats against surgery-induced memory impairments?—Adult and aged rats received three subcutaneous injections of *M. vaccae* or vehicle spaced one week apart (i.e., one injection per week for three weeks). Five days following the final injection of *M. vaccae*, rats underwent a laparotomy or sham surgery. Three days post-surgery rats received a contextual fear conditioning pre-exposure paradigm, to evaluate hippocampal-dependent memory (Barrientos et al., 2012b; Barrientos et al., 2006). This experiment started with an $n = 6$ per group (48 rats total) and three rats were excluded from the final analyses because they developed tumors (1 aged-vehicle-sham, 1 aged-vehicle-laparotomy, and 1 aged-*M. vaccae*-laparotomy). The pre-exposure paradigm separates the construction of the conjunctive representation of the conditioning context from the association of that representation with the shock. This behavioral procedure allows for detection of memory impairments more selective to the hippocampus than does a standard fear-conditioning paradigm (Barrientos et al., 2006). On the first day of the paradigm (3 days post-laparotomy), rats were placed in a novel clear plastic conditioning chamber (26 cm length × 21 cm width × 24 cm height) with a removable floor of stainless steel rods (1.5 mm diameter) that was placed inside a white ice chest that was open to the front. Rats were exposed to the context six times over the course of 6 h during the light phase. The duration of the first context exposure was 5 min and subsequent exposures lasted 40 sec. Three days later, rats were placed in the conditioning context and immediately received a 2 sec 1.5 mA shock (in context for < 5 sec). 24 h following the immediate shock, animals were placed back into the conditioning context or a control context and freezing behavior was assessed, as an index of contextual memory, over a 6-min period. Freezing behavior was scored by a condition-blind observer. 24 h following fear conditioning testing, tissue was collected. This experiment is outlined in Figure 1A.

2.2.2. Experiment 2: Does M. vaccae immunization shift the inflammatory profile of aged rats post-surgery?—Adult and aged rats were immunized with *M. vaccae* or vehicle (3 subcutaneous injections, once per week). Five days following the final injection of *M. vaccae*, rats underwent laparotomy or sham surgery. This experiment started with n = 7 per group; two aged rats were excluded from the final analyses due to tumors (1 aged-vehicle-sham and 1 aged-*M. vaccae* laparotomy) and one adult-*M. vaccae*-laparotomy rat was excluded from RNA analyses due to poor quality RNA (housekeeping gene expression also indicated sample was a significant outlier z = 4.4). Three days post-surgery, hippocampal tissue was collected following a PBS perfusion. This time-point post-surgery was selected for assessment of inflammatory mediators because it corresponds to the time post-surgery when conditioning to the context occurred in *Experiment 1*. Surgery-induced inflammatory mediators can interfere with subsequent memory consolidation.

2.2.3. Experiment 3: Can M. vaccae immunization reduce inflammatory priming in microglia isolated from aged rats?—Aged rats were immunized with *M. vaccae* or vehicle (3 subcutaneous injections, once per week). Five days following the final injection, hippocampal tissue was collected following a PBS perfusion. Highly pure microglia were isolated (see section 2.7.) from whole hippocampus and stimulated *ex vivo*

with lipopolysaccharide (LPS) for 2 h (n = 4/group), which elicits a pro-inflammatory immune response in a concentration-dependent manner (Frank et al., 2006).

2.3. M. vaccae preparation and delivery

Rats were treated with a heat-killed *M. vaccae* suspension as previously described (Frank et al., 2018; Reber et al., 2016). Rats received either subcutaneous injections of 0.1 mg whole heat-killed *M. vaccae* suspension (10 mg/ml sterile stock solution diluted to 1 mg/ml with sterile borate-buffered saline; NCTC 11659 strain, batch ENG 1, provided by Bio Elpida, Lyon, France) or vehicle (sterile borate-buffered saline). Rats received three injections, each spaced 1 week apart (see experimental outline in Fig 1A). No adverse physiological or behavioral consequences of *M. vaccae* were observed: there were no overt signs of sickness behavior and *M. vaccae* did not affect weight gain in adult or aged rats (data not shown).

2.4. Laparotomy procedure

This subcostal laparotomy procedure was developed to model human abdominal exploratory surgery by Martin et al and has previously been used in our laboratory (Barrientos et al., 2012b; Martin et al., 2004; Martin et al., 2005). The surgery was performed under aseptic conditions. Rats were anesthetized with halothane anesthesia, the abdominal region was shaved, and the surgical site was cleaned with 70% EtOH followed by surgical scrub. A 3-cm vertical incision was made through the skin, muscle wall, and abdominal wall approximately 0.5 cm below the lower right rib. The surgical opening and viscera were then manipulated. Approximately 10 cm of intestine was exteriorized and vigorously rubbed for 30 sec. The intestines were then placed back into the rat peritoneal cavity and the muscle and abdominal wall were sutured separately using sterile chromic gut sutures (3-0, PS-2, Ethicon). The skin was closed using reflex clips (9 mm, WPI) and a triple antibiotic ointment was applied (CVS). For the sham procedure, rats were anesthetized, shaved, and prepped in the same manner as laparotomy rats, but no incision was made. Sham and laparotomy procedures occurred in parallel: the halothane anesthesia delivery line was bi-furcated such that a sham rat and laparotomy rat were maintained on the same concentration of anesthesia and exposed to anesthesia for the same duration (~25 min).

2.5. Tissue collection

Animals were given a lethal intraperitoneal injection of sodium pentobarbital (Fatal-plus; 150 mg/kg). After rats were completely unresponsive (as assessed by pedal reflex), they were transcardially perfused with ice-cold phosphate buffered saline (0.9% saline) for 3 min to remove peripheral immune leukocytes from the central nervous system (CNS) vasculature. Brains were rapidly extracted, placed on ice, and the hippocampus was dissected out. Hippocampus was collected given that this brain region is particularly vulnerable to age-associated neuroinflammatory priming (Barrientos et al., 2009). For experiments involving measurement of *in vivo* cytokine mRNA expression, hippocampus was flash frozen in liquid nitrogen and stored at −80 °C. For experiments involving *ex vivo* LPS stimulation of isolated hippocampal microglia, hippocampal microglia were immediately isolated.

2.6. ELISA

Hippocampal samples were sonicated on ice using a tissue extraction reagent (Invitrogen; Catalog #: FNN0071) supplemented with protease inhibitor cocktail (Sigma-Aldrich; Catalog #: P2714). Homogenates were centrifuged (14,000×*g* for 10 min at 4 °C) and supernatants collected and stored at −20 °C. Total protein was quantified using a Bradford assay. An ELISA for rat IL-1β (R&D systems; Catalog #: RLB00) was run according to the manufacturer's instructions and IL-1β protein levels normalized to total protein (pg IL-1β/100 μg total protein).

2.7. Microglia isolations and ex vivo treatments

Hippocampal microglia were isolated using a Percoll density gradient as previously described (Frank et al., 2006). Rats were PBS-perfused, brains were removed, and the hippocampus was dissected out on ice. The hippocampus was homogenized in 3 mL of 0.2% glucose in 1X Dulbecco's Phosphate Buffered Saline (DPBS). The hippocampal homogenate was passed through a 40-μm filter and the filter was rinsed with an additional 2 mL of DPBS. Cells were pelleted in 5-ml falcon tubes at 1000 *g* for 10 min at 22 °C and then the supernatant was poured off. A Percoll (GE Healthcare) gradient was created by re-suspending the pellet in 2 mL of 70% isotonic Percoll (isotonic Percoll consists of 10:1 Percoll:10X DPBS; 100% isotonic Percoll is then diluted with 1X DPBS), followed by a layer of 2 mL 40% isotonic Percoll and topped with 1 mL DPBS. The gradient was spun at 1200 *g* for 30 min at 22 °C with no acceleration or brake. Myelin debris was removed and then microglia were extracted from the 40%/70% interface. Microglia were washed in DPBS and pelleted at 1000 *g* for 10 min at 22 °C. Microglia were resuspended in media (filtered Dulbecco's Modified Eagle Medium (DMEM (Gibco) +10% fetal bovine serum (FBS)) and microglia concentration and viability were determined by trypan blue exclusion. Microglia were plated at a density of 8,000 cells/100 μL in a 96-well v-bottom plate. To assess microglia cytokine responsiveness, cells were challenged *ex vivo* with lipopolysaccharide (LPS; *E. coli* serotype 0111:B4; Sigma-Aldrich) at a concentration of 10 or 100 ng/mL or media alone at 37 °C, 5% CO2. The LPS concentrations, incubation time, and cell density were based on previous publications from our laboratory (Frank et al., 2010; Frank et al., 2006). After 2 h, plates were centrifuged at 1000 *g* for 10 min at 4 °C to pellet cells and wells were aspirated. Cells were washed with 1X DPBS, centrifuged at 1000 *g* for 10 min at 4 °C, and RNA was isolated using a CellsDirect Kit (Invitrogen; Catalog #: 11739-010) according to the manufacturer's instructions.

2.8. qPCR

Rat primers were previously designed using Genbank at the National Center for Biotechnology Information (NCBI), the Operon Oligo Analysis Tool, and the Basic Local Alignment Search Tool at NCBI and obtained from Invitrogen. Primers were designed to span exon/exon boundaries and thus exclude amplification of genomic DNA. Primer specificity was verified by melt curve analysis. Primers included Arg1 (F: CTACCTGCTGGGAAGGAAG and R: GTCCTGAAAGTAGCCCTGTC), β-actin (F: TTCCTTCCTGGGTATGGAAT and R: GAGGAGCAATGATCTTGATC), CD200 (F: CTCTCTATGTACAGCCCATAG and R: GGGAGTGACTCTCAGTACTAT), CD206 (F:

AATGGGTGCCTCCCTGGTTT and R: AGGGTCACCCGTTTTCCAGT), CD3 (F: GCCAGGAAGAGTATGAAGTC and R: GAAATCCTCTAGCACCAGGT), FOXP3 (F: TTATCCAGCCTGCCTCAGAC and R: TAGGAGTTCTGAGCCCTTGG), IL-1β (F: CCTTGTGCAAGTGTCTGAAG and R: GGGCTTGGAAGCAATCCTTA), IL-4 (F: CAACAAGGAACACCACGGAG and R: GGTGCAGCTTCTCAGTGAGT) IL-6 (F: AGAAAAGAGTTGTGCAATGGCA and R: GGCAAATTTCCTGGTTATATCC), IL-10 (F: GGACTTTAAGGGTTACTTGGG and R: AGAAATCGATGACAGCGTCG), and MHCII (F: AGCACTGGGAGTTTGAAGAG and R: AAGCCATCACCTCCTGGTAT). RNA was extracted from hippocampal homogenates using TRIZOL reagent and 2 μg of RNA was reversed transcribed to cDNA using Superscript II (Invitrogen; Catalog #: 18064014) according to the manufacturer's instructions. RNA was isolated from microglia and reversed transcribed to cDNA using SuperScript III CellsDirect cDNA Synthesis System (Invitrogen; Catalog #: 18080-300). PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Qiagen; Catalog # 204145) with a MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Gene expression was run in duplicate and analyzed with the 2^ΔΔCt method relative to β-actin. There were no group differences in β-actin expression.

2.9. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Data were analyzed with StatView and Prism 7 (GraphPad Software, La Jolla, CA). Data were analyzed using analysis of variance (ANOVA) with age, surgery, and *M. vaccae* treatment as the between-subjects factors (2 × 2 × 2). For the *ex vivo* microglia experiment, results were analyzed with 2 × 3 ANOVAs with *M. vaccae* and LPS as the independent variables. *F* values are reported for each ANOVA and serve as the criteria for post hoc analysis (Tukey's HSD). Threshold for statistical significance was set at two-tailed $p < 0.05$.

3. Results

3.1. M. vaccae immunization prevented memory deficits in aged rats following surgery

Young (3 mos) and aged (24 mos) rats received three subcutaneous injections of *M. vaccae* or vehicle, each injection spaced 7 days apart (experiment outlined in Fig 1A). Five days following the final injection of *M. vaccae* rats underwent a laparotomy or sham procedure (described in 2.4). After a 3-day recovery period rats were tested in a pre-exposure fear conditioning paradigm (described in 2.2.1). Freezing behavior is used as an index of memory in this task as freezing is a dominant fear response in rats. There were no differences in baseline freezing (prior to conditioning) between the groups (data not shown, $p > 0.05$). During the testing portion of the fear conditioning paradigm, there was a three-way interaction for freezing behavior in the conditioned context (age × *M. vaccae* × surgery: $F_{1,36} = 5.7$, $p < 0.05$, Fig 1B). In agreement with previous findings, aged vehicle-treated rats that underwent a laparotomy procedure had reduced freezing in the context where they previously received a foot shock (conditioned context), which is suggestive of an impairment in memory (post hoc, $p < 0.05$). Indeed, postoperative aged rats showed a 25% reduction in freezing compared to aged rats that underwent the sham procedure; however, this freezing deficit was completely abolished by *M. vaccae* treatment. Aged rats that were immunized

with *M. vaccae* prior to a laparotomy procedure displayed comparable freezing to aged sham animals (post hoc, $p > 0.05$). There were no differences in freezing behavior in adult rats, or between any of the groups in the novel context (Fig 1B&C). Thus, postoperative memory deficits in aged rats were completely ameliorated by *M. vaccae* immunization.

3.2. M. vaccae immunization altered neuroinflammatory responses in aged rats that underwent a laparotomy

Young and aged rats received three subcutaneous injections of *M. vaccae* or vehicle, each injection spaced seven days apart. Five days following the final injection with *M. vaccae*, rats underwent a laparotomy or sham procedure and tissue was collected three days later (experiment outlined in Fig 2A). Previous work indicates that age-related POCD in rats is dependent on increases in hippocampal IL-1β (Barrientos et al., 2012b). In agreement with these findings, aged rats that received a laparotomy procedure had elevated IL-1β mRNA expression (age × surgery: $F_{1,45} = 5.3$, $p < 0.05$; Fig 2B). The three-way interaction of age × surgery × *M. vaccae* was not statistically significant ($p = 0.1$); however, treatment with *M. vaccae*, reduced IL-1β mRNA (main effect of *M. vaccae*: $F_{1,45} = 6.5$, $p < 0.05$). Hippocampal IL-1β protein was regulated in a similar manner as IL-1β mRNA (Fig 2C). Aged vehicle-treated rats that underwent a laparotomy procedure had enhanced IL-1β protein and *M. vaccae* immunization protected against the increase in IL-1β. Age- and surgery-induced elevations in NFKBIA mRNA expression (age: $F_{1,45} = 13.9$, surgery: $F_{1,45} = 8.8$, $p < 0.05$) were also ameliorated by *M. vaccae* pretreatment (*M. vaccae*: $F_{1,45} = 4.3$, $p < 0.05$; Fig 2D).

M. vaccae can buffer against the pro-inflammatory effects of stress by upregulating anti-inflammatory pathway genes in the periphery (IL-10 and transforming growth factor beta [TGFβ]) and central nervous system (IL-4) (Reber et al, 2016; Frank et al, 2018). Thus, here we evaluated whether *M. vaccae* immunization induces an anti-inflammatory immunophenotype in the aged brain. Overall, IL-10 mRNA was expressed at a low level and was not detectable in a number of samples (spread equally throughout the groups). In PCR samples that did amplify before 35 cycles, there was an age-related reduction in IL-10 mRNA expression ($F_{1,34} = 9.1$, see Table 1). However, *M. vaccae* did not significantly upregulate IL-10 mRNA expression. TGF-β was not significantly regulated by age, surgery, or *M. vaccae* treatment (Table 1). In contrast, IL-4 mRNA expression was suppressed in the hippocampus of aged rats. *M. vaccae* treatment was protective against age-associated decrements in IL-4 (age × *M. vaccae*: $F_{1,45} = 7.2$, $p < 0.05$; Fig 2E): *M. vaccae* increased IL-4 expression in the hippocampus of aged, but not young adult rats (post hoc, $p < 0.05$).

IL-4 induces anti-inflammatory (M2) polarization in peripheral macrophages (Koning et al., 2010) as well as cluster of differentiation 200 (CD200) in the CNS (Lyons et al., 2007a), which inhibits pro-inflammatory activation in microglia through its cognate receptor cluster of differentiation 200 receptor 1 (CD200R1) expressed on microglia (Gorczynski et al., 2004). Thus, we also evaluated several markers typical of M2 cells (arginase 1 (Arg1) and cluster of differentiation 206 (CD206)) and CD200. Arg1 and CD206 were reduced in aged rats and significantly upregulated by *M. vaccae* treatment (age × *M. vaccae*, $p < 0.05$; Fig 2F and Table 1). There were also age-related reductions in CD200 (age: $F_{1,45} = 7.7$, $p < 0.05$),

but no compensation by treatment with *M. vaccae* ($p > 0.05$; Table 1). There was no effect of surgery or three-way interaction on Arg1, CD206, or CD200 gene expression. Of note, tissue for gene analyses was collected 8 days after the final *M. vaccae* injection so it is possible that upregulation of additional markers of alternative activation might occur at acute (earlier) post-injection time points.

To determine whether *M. vaccae* had persistent effects on neuroinflammatory phenotype, hippocampi were examined from rats that underwent fear conditioning (13 days following the final injection of *M. vaccae*). At this later time post-*M. vaccae* and -laparotomy, IL-4 and Arg1 were also significantly upregulated in the hippocampus of *M. vaccae* immunized rats ($F1,36 = 7.6$, $p < 0.05$; Table 1). There were no differences in IL-1β at this later time point ($p > 0.05$, Table 1). Thus, *M. vaccae* treatment in aged rats elicits an anti-inflammatory shift in the CNS milieu that persists for at least ~2 weeks post-immunization.

3.3. Aged rats increased T cell markers in the hippocampus

The peripheral anti-inflammatory effects of *M. vaccae* have been largely attributed to effects on T cells (Reber et al., 2016). However, T cells are not routinely detected in the brain parenchyma as access is tightly regulated by the blood-brain barrier. Here, we show that mRNA expression for the pan T cell marker, CD3, is upregulated in the hippocampus of aged rats and reduced by *M. vaccae* immunization (age × *M. vaccae* $F_{1,45} = 8.6$, < 0.05; Fig 3A). CD4, a marker for helper T cells, was upregulated in the hippocampus of aged rats (age: $F_{1,45} = 90.9$, $p < 0.05$; Fig 3B). Furthermore, Forkhead Box P3 (FOXP3) mRNA expression, which directs development and function of regulatory T cells, was increased by *M. vaccae* treatment in aged rats (age: $F_{1,45} = 5.2$, *M. vaccae*: $F_{1,45} = 4.7$, $p < 0.05$; Fig 3C). This may indicate that *M. vaccae* treatment shifts the population of T cells gaining access to the CNS.

3.4. M. vaccae immunization dampened inflammatory responses of aged microglia ex vivo

Microglia are considered the predominant innate immune cell of the CNS (Ransohoff and Perry, 2009) and a key cellular substrate of aging-induced neuroinflammatory priming (Barrientos et al., 2012a). Thus, the present experiment examined whether *in vivo M. vaccae* immunization in aged rats reduces *ex vivo* microglia reactivity. To test this possibility, microglia were isolated from the hippocampus of aged rats five days after the final injection of *M. vaccae* and directly exposed to an immune challenge (outlined in Fig 4A). It is important to note here that in the findings presented thus far, laparotomy served as an immune challenge *in vivo.* In the present experiment, microglia were directly exposed to an immune challenge *ex vivo* to determine whether *M. vaccae* treatment *in vivo* blunts microglia priming. In this case, LPS served as the immune challenge instead of laparotomy given our prior findings that the microglial pro-inflammatory response to LPS *ex vivo* is potentiated in aged animals (Frank et al., 2010). Also, it is important to note that young animals were not included here given the lack of an *M. vaccae* effect on anti-inflammatory processes in these animals. Microglia were plated *ex vivo* and challenged with media alone (0 ng LPS control) or LPS (10 and 100 ng). *M. vaccae* immunization blunted aged microglia reactivity to LPS as indicated by IL-1β mRNA expression (*M. vaccae* × LPS: $F_{2,18} = 3.7$, $p < 0.05$; Fig 4B) and IL-6 mRNA expression (LPS: $F_{2,18} = 30.7$, *M. vaccae*: $F_{1,18} = 8.7$, $p <$

0.05; Fig 4C). *M. vaccae* also reduced NFKBIA increases caused by LPS treatment (*M. vaccae* × LPS: $F_{2,18} = 4.6$, $p < 0.05$, Fig 4D).

4. Discussion

Aged individuals are vulnerable to developing cognitive impairments following surgery (Barrientos et al., 2012b; Monk et al., 2008; Steinmetz et al., 2009) and cognitive decline post-surgery appears dependent on a pro-inflammatory neuroenvironment (Barrientos et al., 2012b). Indeed, consistent with our prior findings (Barrientos et al., 2012b), the present results demonstrate that a laparotomy procedure induced impairments in hippocampal-dependent memory, concomitant with increased hippocampal pro-inflammatory cytokines in aged, but not young rats. This finding is consistent with a number of prior reports demonstrating that the neuroimmune microenvironment of aged animals becomes sensitized to diverse inflammatory stimuli including *E. coli* infection and traumatic brain injury (Barrientos et al., 2015). For this reason, treatment strategies that induce a prolonged desensitization of the neuroimmune microenvironment might be particularly effective at mitigating the heightened neuroinflammatory effects of immune challenges in aged animals. Here we focused on a microbial-based treatment strategy (*M. vaccae*), which buffers against pro-inflammatory conditions in the periphery (Reber et al., 2016) and has prolonged anti-inflammatory effects (~12 weeks) (Zuany-Amorim et al., 2002). It is important to note that the effects of *M. vaccae* on neuroimmune processes have largely been unexplored. Thus, here we tested the hypothesis that a microbial treatment prior to surgery could re-direct the CNS immune system towards an anti-inflammatory phenotype and prevent surgery-induced neuroinflammatory increases and cognitive dysfunction. Our findings suggest that *M. vaccae* pretreatment protects against surgery-induced cognitive impairments in aged rats. Furthermore, *M. vaccae* treatment shifted the aged hippocampus from a pro-inflammatory milieu (increased IL-1β and NFKBIA) towards an anti-inflammatory phenotype (reduced IL-1β and NFKBIA and increased IL-4 and Arg1). Our results also indicate a possible role for microglia and T cells in mediating the benefits of *M. vaccae* treatment.

M. vaccae may abrogate neuroinflammatory priming in aged rats through upregulating IL-4 signaling. In agreement with previous findings, aged rats had reduced hippocampal IL-4 mRNA expression (Maher et al., 2005; Nolan et al., 2005) and IL-4 was further suppressed in aged rats by a surgical challenge (Li et al., 2017). Reductions in hippocampal IL-4 in aged rats have previously been associated with cognitive deficits and impairments in LTP (Li et al., 2017; Lyons et al., 2007b; Maher et al., 2005; Nolan et al., 2005). Central administration of IL-4 is able to reverse inflammation-associated deficits in aged animals (Li et al., 2017; Lyons et al., 2007b; Maher et al., 2005; Nolan et al., 2005). Here, we show that *M. vaccae* treatment induced a prolonged (8 and 13 days post-*M. vaccae*) upregulation in IL-4 mRNA expression in the hippocampus of aged rats. Importantly, IL-4 acts as an anti-inflammatory agent on innate immune cells including microglia and can help resolve an inflammatory response (Gadani et al., 2012). IL-4 can induce an alternative activation state (M2) in macrophages and microglia (Gadani et al., 2012). In support of this, Arg1, which is a traditional marker for M2 macrophages/microglia, was also elevated by *M. vaccae*.

Consistent with its induction of an anti-inflammatory milieu (i.e., increased IL-4) in the hippocampus of aged animals, *M. vaccae* treatment also abrogated the potentiating effect of surgery on pro-inflammatory factors in the aged hippocampus. NFKBIA expression was elevated in aged rats that underwent surgery but reduced by *M. vaccae* treatment. NFKBIA expression is a general indicator of activity of the canonical inflammatory transcription factor NF-κB, such that NFKBIA is induced by NF-κB to provide negative feedback inhibition of NF-κB function (Sun et al., 1993). In parallel, IL-1β mRNA and protein expression were upregulated in the hippocampus of aged rats compared to young rats 3 days following laparotomy. *M. vaccae* treatment blocked the surgery-induced protein and mRNA increase of this pro-inflammatory cytokine in aged animals. Pro-inflammatory changes following laparotomy, and specifically elevations in IL-1β, are essential for cognitive deficits in this laparotomy model: blocking IL-1β signaling with an intracisterna magna (ICM) injection of IL1 receptor antagonist (IL1-RA) can prevent cognitive deficits in aged rats (Barrientos et al., 2012b). The laparotomy-elicited pro-inflammatory response likely resolves by 8 days post-surgery as the elevation in hippocampal IL-1β mRNA expression was not evident in tissue collected 8 days following laparotomy in rats that underwent behavioral testing. It is possible, however, that the stress of behavioral testing disrupted IL-1β signaling at this time point. Here, we focused on what is considered the “master” pro-inflammatory cytokine IL-1β (Basu et al., 2004), which is thought to set in motion a cascade of downstream inflammatory processes. Our previous work indicates that aging-induced priming is not restricted to IL-1β signaling, but more generally increases a number of pro-inflammatory pathways in response to immune stimulation (Fonken et al., 2016). This is consistent with the notion that IL-1β signaling is a “gatekeeper” of inflammation (Dinarello, 2011).

M. vaccae could facilitate anti-inflammatory changes in the CNS via several potential mechanisms. First, *M. vaccae* could communicate anti-inflammatory signals through the vagus nerve. A precedent for this idea extends from findings that inflammatory mediators such as cytokines are detected by afferent vagal neurons in the nodose and jugular ganglia that signal to the nucleus of the solitary tract and other brain stem structures (reviewed in (Pavlov and Tracey, 2012)). This afferent signal is essential for the communication of pro-inflammatory signals to the CNS (Watkins et al., 1995). Whether anti-inflammatory signals are similarly communicated, however, is less well established. Interestingly, the vagus nerve also mediates gut-brain axis signaling (Breit et al., 2018). Subcutaneous injections of *M. vaccae* induce changes in the gut microbiota, suggesting that *M. vaccae* may also signal the vagus via this indirect pathway (Reber et al., 2016). Second, it is possible that *M. vaccae* blunts the peripheral inflammatory response at the surgical site and thus prevents the communication of an enhanced signal to the brain. This explanation is unlikely, however; aged rats tend to exhibit dampening of peripheral immune responses with age (Rawji et al., 2016). Previous research has demonstrated that inflammatory priming is specific to the CNS in aged rats (Barrientos et al., 2009). Third, *M. vaccae*-derived metabolites, such as triacylglycerols, long-chain saturated fatty acid polyesters, or their fatty acid derivatives (Agusti et al., 2008) may cross the blood-brain barrier. Fourth, peripheral immune cells, such as macrophages or T cells, may traffic into the CNS or signal through the meningeal compartment. Although T cells do not typically access the CNS under healthy conditions,

there is evidence that aged animals exhibit a gradual enhancement in T cell homing to the brain (Gemechu and Bentivoglio, 2012). Indeed, here we show that the pan T cell marker, CD3 was upregulated in the aged hippocampus and reduced by *M. vaccae* immunization. CD4, which is a glycoprotein found on the surface of helper T cells was also upregulated in the hippocampus of aged rats (CD4 expression was not altered by *M. vaccae*). Furthermore, *M. vaccae* may shift the phenotype of the T cell population as *M. vaccae* increased hippocampal expression of FOXP3, a marker of regulatory T cells (Tregs). Importantly, the peripheral anti-inflammatory effects of *M. vaccae* require Tregs (Reber et al., 2016). T cells can modulate innate immune cells in the CNS (Walsh et al., 2014; Xie et al., 2015) and redirecting the population of T cells that access the CNS promotes a reparative environment in other contexts. Future studies could explore how peripheral *M. vaccae* alters T cell density and phenotype in the CNS.

Microglia likely underlie exaggerated immune responses in the aged brain, as aged microglia *ex vivo* show amplified responses to inflammatory stimuli (Fonken et al., 2016; Frank et al., 2010). The present results demonstrate that *M. vaccae*, as compared to vehicle treatment, induced a considerable reduction in the pro-inflammatory response of microglia isolated from aged rats. These results suggest that *M. vaccae* immunization shifts the balance of neuroimmune microenvironment towards an anti-inflammatory milieu resulting in desensitization of microglia to pro-inflammatory stimuli. Further, these findings are consistent with the induction of IL-4 by *M. vaccae* in the aged hippocampus. For instance, microglia exposed to IL-4 take on a more reparative “alternatively activated” phenotype (Butovsky et al., 2006; Kigerl et al., 2009). Interestingly, microglia from aged mice are not as responsive to *ex vivo* IL-4 treatment (Fenn et al., 2012) and previous work suggests that microglia isolated from aged mice have impaired IL-4 signaling following infection or injury (Fenn et al., 2014; Fenn et al., 2012). We found that *M. vaccae* upregulated hippocampal IL-4 in aged rats for at least 13 d post-treatment, and that hippocampal microglia from *M. vaccae*-treated aged rats were less responsive to inflammatory stimulus *ex vivo*. Thus, *M. vaccae* treatment may be a novel strategy to rescue decrements in the IL-4 system in aged animals.

One limitation to this study is that it did not include female rats as aged F344xBN female rats were not available through the NIA colony. There are sex differences in the neuroimmune system (Osborne et al., 2018), including sex differences in microglia priming in other contexts such as stress (Fonken et al., 2018a). Furthermore, mild cognitive impairments develop more rapidly in women (Lin et al., 2015) and women are at great risk for developing Alzheimer’s disease (Altmann et al., 2014). Thus, determining whether *M. vaccae* is protective against cognitive decline in females is an important future direction.

5. Conclusions

Taken together, these results indicate that *M. vaccae* pretreatment can prevent pathological neuroinflammation and cognitive impairments in aged rats that undergo a surgical challenge. In aged rats, prior *M. vaccae* immunization was able to rescue surgery-elicited memory deficits. In parallel, *M. vaccae* boosted expression of anti-inflammatory and regulatory T cell markers in the aged hippocampus, suggesting that it induces a persistent beneficial immune

shift in the CNS. Further, microglia from *M. vaccae*-treated aged hippocampus displayed dampened neuroinflammatory priming.

Our data highlight the beneficial immunomodulatory role of *M. vaccae* in a model of aging-elicited neuroinflammatory priming. *M. vaccae* could improve other conditions caused or exacerbated by neuroinflammatory priming, and future studies could address whether delivering *M. vaccae* after neuroinflammatory activation can still be protective. In addition, our data suggest that neuroinflammatory priming with age could be improved by exploring other potential microbial treatments (Williamson et al., 2016). One remarkable strength of the treatment regimen employed herein is that the delivery route is minimally invasive (subcutaneous), yet able to modulate neuroinflammation in the hippocampus and ameliorate related behavioral deficits. Future studies should reveal whether *M. vaccae* and other micro-/macroorganism treatments improve neuroinflammatory pathology, how this peripheral-to-central signaling occurs, and whether these treatments are viable immunomodulators in humans.

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Abbreviations

Arg1	arginase 1
CD200	cluster of differentiation 200
CD200R	cluster of differentiation 200 receptor
CD206	cluster of differentiation 206, also known as mannose receptor (MR)
CNS	central nervous system
DMEM	Dulbecco’s Modified Eagle Medium
DPBS	Dulbecco’s Phosphate Buffered Saline
FBS	fetal bovine serum
FOXP3	forkhead box P3
ICM	intracisterna magna
IL	interleukin
IL1-RA	IL1 receptor antagonist
LPS	lipopolysaccharide
LTP	long-term potentiation

NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
PBS	phosphate-buffered saline
POCD	postoperative cognitive dysfunction
TGFβ	transforming growth factor beta

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Highlights

- Aged, but not young rats, show post-operative learning/memory deficits.
- Prophylactic immunization with *M. vaccae* prevents cognitive deficits in aged rats.
- *M. vaccae* likely prevents cognitive impairments by shifting the neuroimmune environment.
- *M. vaccae* immunization may signal the CNS via T cells.

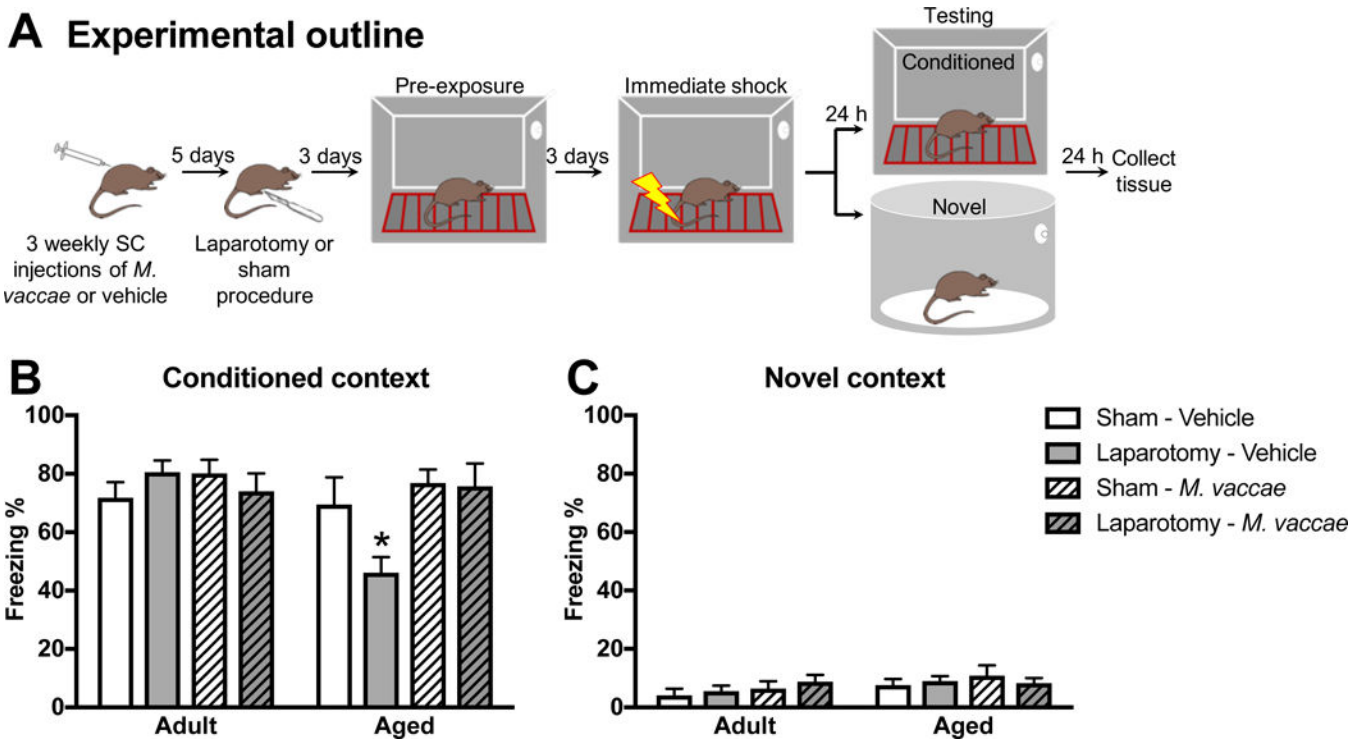

Figure 1. *M. vaccae* prevents laparotomy-induced cognitive impairments in aged rats

(A) Experimental outline: aged and young rats received subcutaneous *M. vaccae* injections once per week for three weeks. Five days following the final injection, rats underwent a laparotomy or sham procedure. Three days post-surgery, rats underwent training in a pre-exposure fear-conditioning paradigm. Freezing behavior in the (B) conditioned and (C) novel context of the fear-conditioning paradigm is presented as percent of total time (6 min) freezing (Freezing %). Results were analyzed using a 2 × 2 × 2 ANOVA with age, surgery, and *M. vaccae* treatment as between-subjects factors (n = 5-6/group). Data are expressed as mean ± SEM. *differs from all other groups, $p < 0.05$.

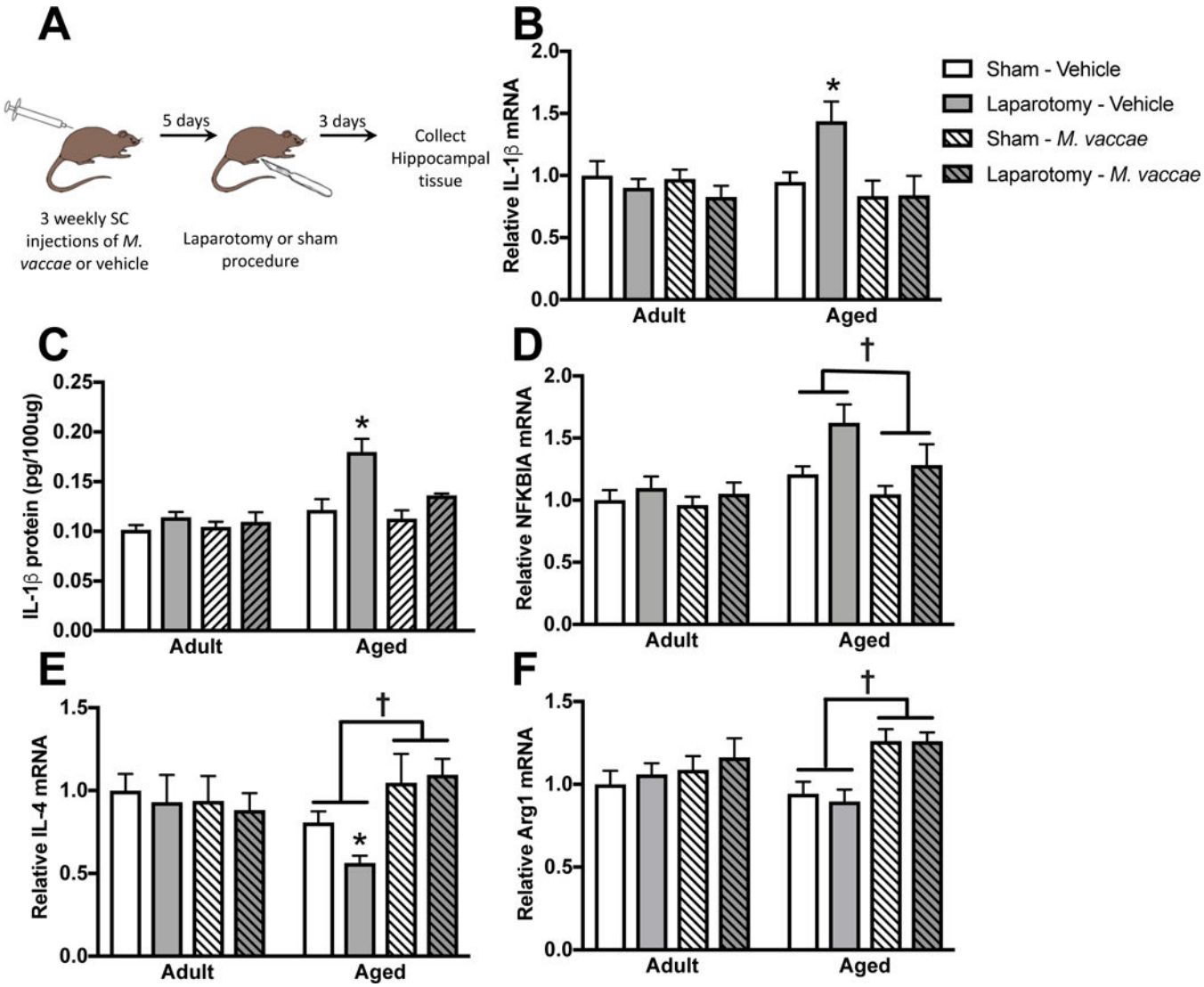

Figure 2. The hippocampal pro-inflammatory environment in aged rats was abrogated by *M. vaccae* treatment

(A) Experimental outline. Hippocampal IL-1β (B) gene and (C) protein expression were elevated in aged rats that underwent a laparotomy procedure and reduced by *M. vaccae* treatment. (D) NFKBIA was also elevated by surgery in aged rats and reduced by *M. vaccae*. *M. vaccae* treatment upregulated (E) IL-4 and (F) Arginase1 mRNA expression. Results were analyzed using a 2 × 2 × 2 ANOVA with age, surgery, and *M. vaccae* treatment as between-subjects factors (n = 6-7/group). Data are expressed as mean ± SEM. *differs from all other groups, †effect of *M. vaccae*, $p < 0.05$.

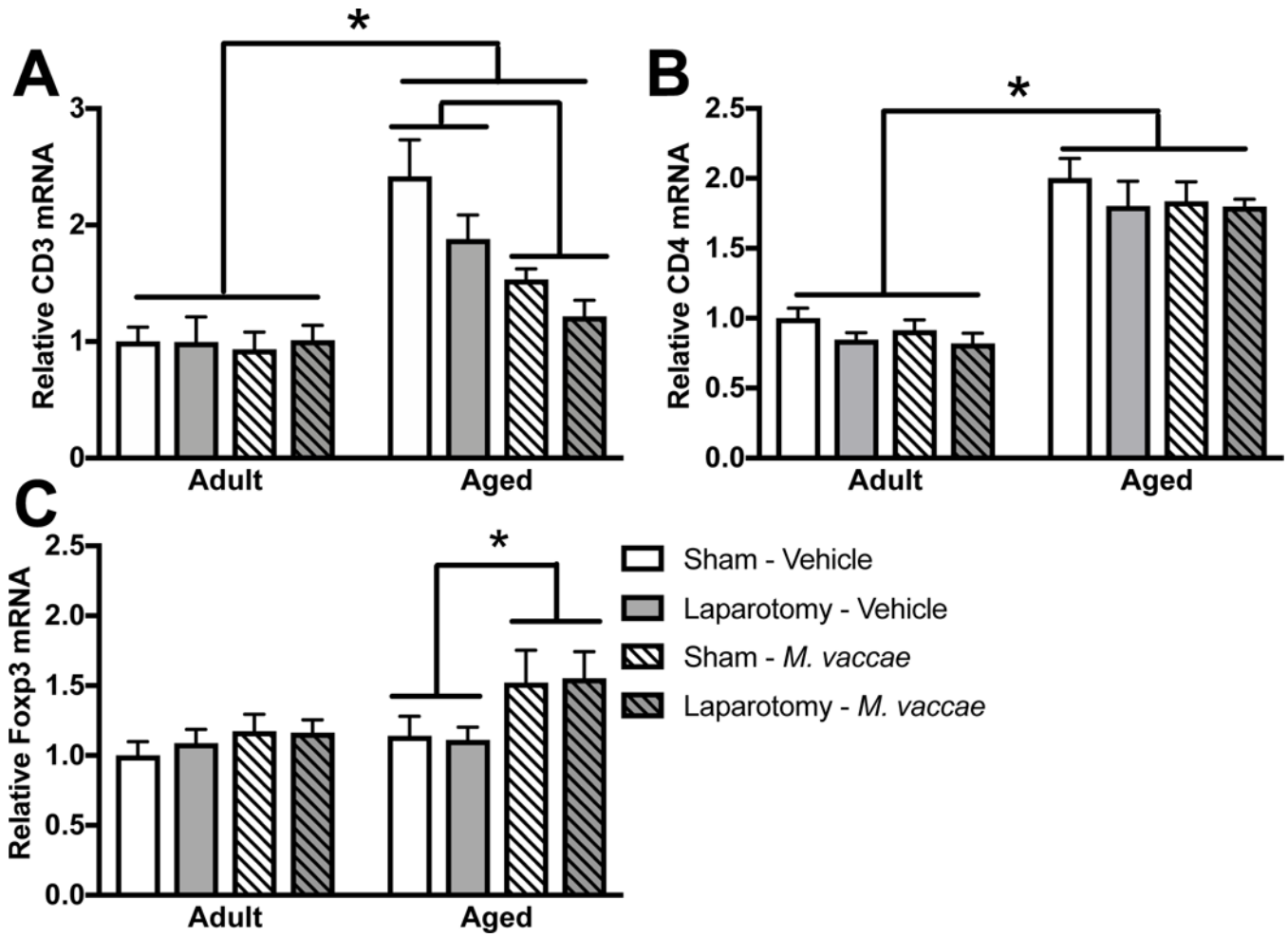

Figure 3. T cell markers are regulated by aging and *M. vaccae*

(A) CD3 mRNA expression was upregulated in the hippocampus of aged as compared to young rats and reduced by *M. vaccae* immunization. (B) CD4 mRNA expression was upregulated in the aged rat hippocampus and (B) Foxp3 mRNA expression was elevated in aged rats by *M. vaccae* treatment. Results were analyzed using a 2 × 2 × 2 ANOVA with age, surgery, and *M. vaccae* treatment as between-subjects factors (n = 6-7/group). Data are expressed as mean ± SEM. *$p < 0.05$.

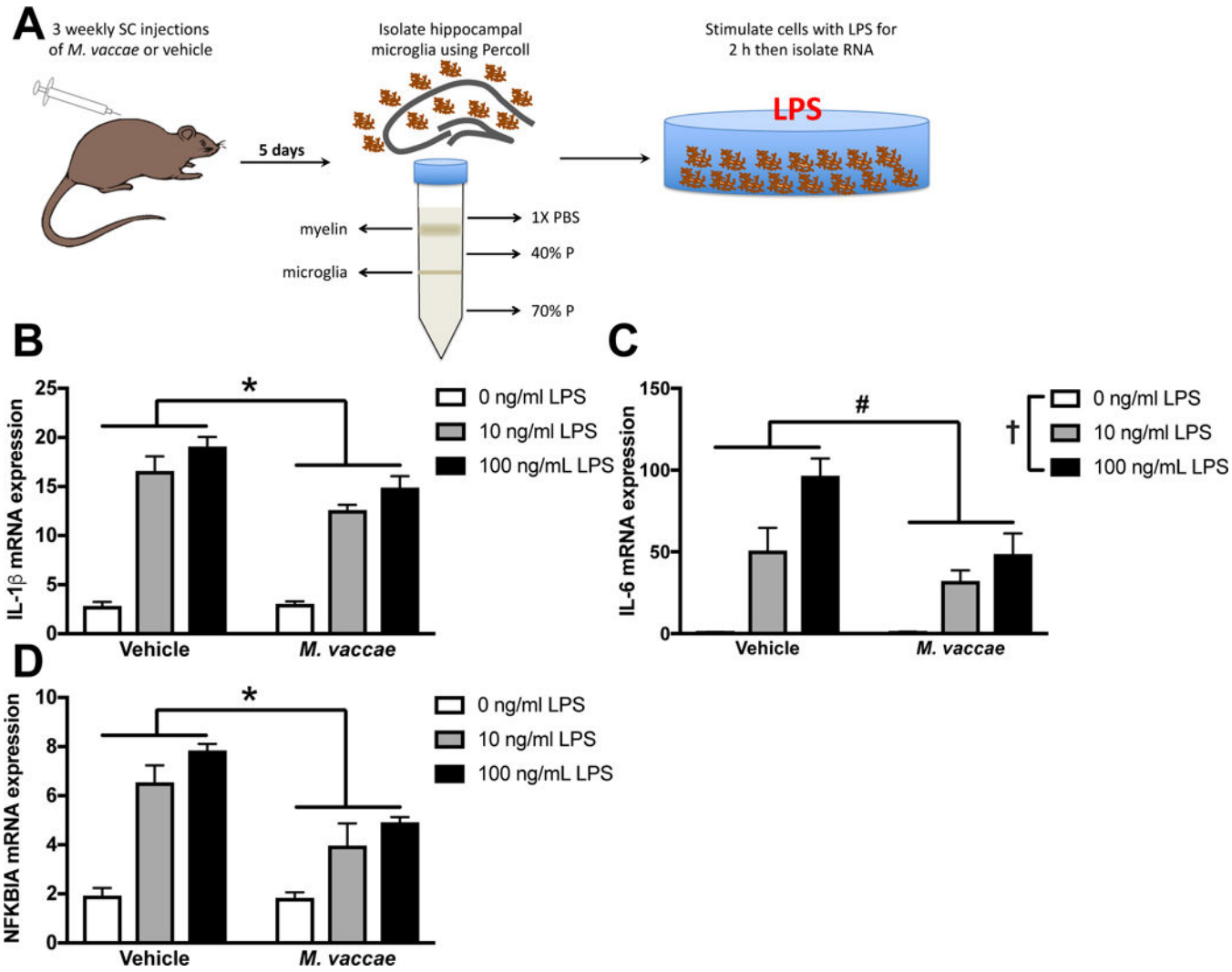

Figure 4. Microglia isolated from aged rats are less inflammatory following *in vivo M. vaccae* treatment

(A) Experimental design: aged rats received subcutaneous *M. vaccae* injections once per week for three weeks. Five days following the final injection, microglia were isolated from the hippocampus and stimulated with LPS. (B) IL-1β, (C) IL-6, and (D) NFKBIA mRNA expression were reduced in microglia isolated from aged *M. vaccae*-treated rats compared to vehicle-treated rats. Results were analyzed using a 2 × 3 ANOVA with age and LPS as the independent variables (n = 4/group). Data are expressed as mean ± SEM. *LPS × *M. vaccae* interaction, $^{\#}$main effect of *M. vaccae*, †main effect of LPS; $p < 0.05$ in all cases.

Table 1

Relative gene expression. Gene expression was run in duplicate and analyzed with the 2^ΔΔCt method relative to β-actin.

Group	Adult				Aged			
	Vehicle		*M. vaccae*		Vehicle		*M. vaccae*	
	Sham	Laparotomy	Sham	Laparotomy	Sham	Laparotomy	Sham	Laparotomy
Gene	**Three days post-laparotomy (behaviorally naïve rats)**							
Arg1	1.00 ± 0.08	1.06 ± 0.07	1.09 ± 0.08	1.16 ± 0.11	0.94 ± 0.07	0.9 ± 0.07	1.26 ± 0.07	1.26 ± 0.05
CD200L	1.00 ± 0.08	1.02 ± 0.07	0.98 ± 0.06	0.99 ± 0.11	0.82 ± 0.06	0.79 ± 0.05	0.94 ± 0.06	1.03 ± 0.12
CD206	1.00 ± 0.09	0.81 ± 0.09	0.88 ± 0.15	0.94 ± 0.11	0.59 ± 0.04	0.49 ± 0.06	0.69 ± 0.04	0.75 ± 0.08
CD-4	1.00 ± 0.07	0.85 ± 0.05	0.92 ± 0.07	0.82 ± 0.07	2.00 ± 0.14	1.80 ± 0.17	1.8 ± 0.14	1.80 ± 0.05
IL-4	1.00 ± 0.10	0.93 ± 0.16	0.94 ± 0.15	0.88 ± 0.10	0.81 ± 0.07	0.56 ± 0.04	1.05 ± 0.17	1.10 ± 0.10
IL-10	1.00 ± 0.17	0.79 ± 0.16	1.02 ± 0.19	0.96 ± 0.31	0.50 ± 0.15	0.43 ± 0.11	0.70 ± 0.13	0.62 ± 0.23
IL-1β	1.00 ± 0.12	0.90 ± 0.07	0.97 ± 0.08	0.83 ± 0.09	0.95 ± 0.08	1.44 ± 0.15	0.83 ± 0.13	0.84 ± 0.16
Foxp3	1.00 ± 0.10	1.09 ± 0.10	1.18 ± 0.12	1.16 ± 0.09	1.14 ± 0.14	1.11 ± 0.09	1.52 ± 0.23	1.55 ± 0.19
NFKBIA	1.00 ± 0.08	1.10 ± 0.10	0.96 ± 0.07	1.05 ± 0.09	1.21 ± 0.06	1.62 ± 0.15	1.05 ± 0.07	1.28 ± 0.17
MHCII	1.00 ± 0.12	0.73 ± 0.08	0.95 ± 0.14	0.73 ± 0.07	4.41 ± 0.29	3.98 ± 0.48	2.94 ± 0.19	2.84 ± 0.37
TGF-β	1.00 ± 0.08	0.84 ± 0.06	0.94 ± 0.06	0.82 ± 0.14	1.04 ± 0.04	0.93 ± 0.06	0.89 ± 0.05	0.97 ± 0.05
Eight days post-laparotomy (behavior rats)								
Arg1	1.00 ± 0.06	0.81 ± 0.09	0.90 ± 0.11	0.80 ± 0.09	0.71 ± 0.08	0.69 ± 0.06	0.90 ± 0.07	1.07 ± 0.07
IL-4	1.00 ± 0.17	1.02 ± 0.07	1.19 ± 0.14	1.06 ± 0.10	0.71 ± 0.09	0.61 ± 0.10	1.06 ± 0.11	1.04 ± 0.07
IL-1β	1.00 ± 0.14	1.19 ± 0.09	1.07 ± 0.08	1.10 ± 0.05	1.11 ± 0.14	0.95 ± 0.13	1.13 ± 0.14	0.87 ± 0.09