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Brain Behav Immun. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:
Brain Behav Immun. 2015 January ; 0: 60–67. doi:10.1016/j.bbi.2014.07.004.

The role of hepatic & splenic macrophages in *E. coli*-induced memory impairments in aged rats

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Abstract

Bi-directional communication between the peripheral and central nervous systems has been extensively demonstrated. Aged rats exhibit a prolonged proinflammatory response in the hippocampus region of the brain following a peripheral bacterial infection, and this response in turn causes robust memory declines. Here we aimed to determine whether hepatic or splenic macrophages play a role in the maintenance of this central response. Proinflammatory cytokines measured in liver and spleen four days following an *E. coli* infection revealed a potentiated proinflammatory response in liver, and to a lesser extent in spleen, in aged relative to young rats. To determine whether this potentiated response was caused by impaired bacterial clearance in these organs, *E. coli* colony forming units in liver and spleen were measured 4 days after infection, and there were no difference between young and aged rats in either organ. No *E. coli* was detected in the hippocampus, eliminating the possibility that the aged blood brain barrier allowed *E. coli* to enter the brain. Depletion of hepatic and splenic macrophages with clodronate-encapsulated liposomes effectively eliminated the proinflammatory response to *E. coli* at four days in both organs. However, this treatment failed to reduce the proinflammatory response in the hippocampus. Moreover, depletion of peripheral macrophages from liver and spleen did not prevent *E. coli*-induced memory impairment. These data strongly suggest that hepatic and splenic macrophages do not play a major role in the long-lasting maintenance of the proinflammatory response in the hippocampus of aged rats following a bacterial infection, or the memory declines that this response produces.

Introduction

Following a peripheral immune challenge, aged rats show a precipitous decline in the formation of long-term memories that depend on an intact hippocampus (Barrientos et al.,

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The authors declare no competing financial interests.

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2006; Chapman et al., 2010). Furthermore, this memory impairment is causally associated with a potentiated and protracted proinflammatory cytokine response in the hippocampus region of the brain (Barrientos et al., 2009b; Frank et al., 2010b). To develop truly effective therapies aimed at reducing this potentiated response in the hippocampus and thus preventing memory declines, it is critical to discover the original distal source of the persistent cytokine production. Several years ago we began to approach this issue by measuring proinflammatory cytokines in various regions of the brain (hippocampus, hypothalamus, prefrontal cortex, and parietal cortex) and in the periphery (spleen and serum) at various time points from two hours to two weeks following an *E. coli* infection in young and aged rats (). Those data revealed that the aged hippocampus was unique in its potentiated and prolonged cytokine profile, as none of the other brain regions examined exhibited an elevated response compared to young adult rats. Serum and spleen samples exhibited the expected elevations in the proinflammatory cytokine interleukin-1 beta (IL-1β) in response to *E. coli*, but these elevations were not significantly different between the age groups. Together, these findings led us to conclude that the protracted cytokine profile in the hippocampus of aged rats was generated and maintained from within the brain, and not from a peripheral source. Further strengthening this conclusion, data from our laboratory, as well as others, demonstrated that isolated hippocampal microglia from aged rodents are primed, or sensitized, such that they produce a potentiated pro-inflammatory cytokine response, compared to that of young adults, when challenged with an inflammatory stimulus (Henry et al., 2009; Frank et al., 2010a). However, compelling findings from several early and recent studies that highlight the critical role that liver macrophages (Kupffer cells) play in signaling the brain following an inflammatory insult originating in either the periphery or the brain, led us to explore whether liver macrophages might not play a role in age-related neuroinflammation and memory impairment following peripheral infection (Blatteis et al., 2004; Campbell et al., 2007; Campbell et al., 2008). Thus, the present study aimed to determine whether the prolonged cytokine response exhibited in the hippocampus is a direct result of a prolonged proinflammatory signal generated by hepatic or splenic macrophages.

Materials and Methods

Subjects

Subjects were male F344xBN F1 rats obtained from Harlan and Charles River colonies maintained for the National Institute on Aging. Upon arrival at our facility, aged rats were 24 mos. old and weighed approximately 550 g. Young adult rats were 3 mos. old and weighed approximately 275 g. All rats were age-matched and housed 2 to a cage (52 L × 30 W × 21 H, cm). The animal colony was maintained at 22±1° C on a 12-h light/dark cycle (lights on at 07:00 h). All rats were allowed free access to food and water and were given at least 1 week to acclimate to colony conditions before experimentation began. All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

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Immune challenge

To examine the proinflammatory response from the liver and spleen of young and aged rats following an immune challenge, rats received an intraperitoneal (i.p.) injection of either live *Escherichia coli* (*E. coli*), or vehicle, and euthanized 4 days later. Four days was chosen as a measure of "long-lasting" inflammation based on our prior work demonstrating that proinflammatory cytokines in the hippocampus of infected aged rats continued to be significantly elevated compared to young rats, whereas cytokine levels in infected young rats have returned to baseline levels (Barrientos et al., 2009b). One day prior to administering the bacteria, stock *E. coli* cultures (ATCC #15746; American Type Culture Collection, Manassas, VA) were thawed and cultured overnight (15-20 hr) in 40 mL of brain-heart infusion (BHI; DIFCO Laboratories, Detroit, MI) in an incubator (37°C). The number of bacteria in cultures was quantified by extrapolating from previously determined growth curves. Cultures were then centrifuged for 15 min at 4°C, 3000 RPM, supernatants discarded, and bacteria resuspended in sterile phosphate buffered saline (PBS) to achieve a concentration of 1.0×10^{10} colony forming units (CFU). A volume of 250 μL was injected i.p. to achieve a final concentration of 2.5×10^{9} CFU. Vehicle-treated rats received an injection of sterile PBS of an equal volume (250 μL).

Tissue dissection and detection of proinflammatory cytokines

Four days following the *E. coli* or vehicle injection, animals were anesthetized with 50mg/kg of sodium pentobarbital and cardiac perfused with 0.9% ice-cold saline. Spleen and liver were collected from the periphery. Hippocampus was dissected from brain. All dissections were performed on an ice-cold frosted glass plate and tissues quickly frozen in liquid nitrogen. Tissue samples were stored at -80°C until the time of sonication. To prepare the tissues for the assays, 0.5 mL (spleen), 1.0 mL (liver), or 0.3 mL (hippocampus) of a sonication buffer containing 50 mM Tris base and a cocktail enzyme inhibitor (100 mM amino-n-caproic acid, 10 mM EDTA, 5 mM benzamidine HCl, and 0.2 mM phenylmethyl sulfonyl fluoride) was added to each sample. Each tissue was mechanically sonicated for ~20 sec using an ultrasonic cell disrupter (Fisher Scientific, Pittsburgh, PA), centrifuged at $10{,}000 \times g$ at 4°C for 10 min, and supernatants removed and stored at 4°C until ELISA was performed. Liver samples were centrifuged a second time, and these second supernatants were used. Bradford protein assays were also performed to determine total protein concentrations in each sample. IL-1β, IL-6, and TNFα cytokine protein levels were determined using commercially available multiplex ELISA kits (Aushon Biosystems, Billerica, MA). For experiment 3, IL-1β was assayed using a single ELISA (R&D Systems, Minneapolis, MN). The assays were performed according to the manufacturer's instructions. Both spleen and liver samples were run at a 10-fold dilution, hippocampus samples were run at a 2-fold dilution. The sensitivity of the multiplex assay is 6.25 pg/mL for IL-1β, 12.5 pg/mL for IL-6, and 3.125 pg/mL for TNFα. The sensitivity of the R&D IL-1β assay is < 5 pg/mL. The concentration of each cytokine is presented as pg/100 ug of total protein.

Bacterial Clearance

To determine whether young and old rats clear bacteria from spleen and liver differently, we conducted a bacterial clearance experiment. To confirm that bacteria do not enter the

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hippocampus, we also included hippocampus in this experiment. Four days following an i.p. *E. coli* injection, both young and old rats were anesthetized with 50mg/kg of sodium pentobarbital. Rats were then transcardially perfused with cold 0.9% saline. Spleens (1g), livers (1g), and one hemisphere of hippocampus were dissected out, and homogenized in 3 mL PBS with a hand-held glass homogenizer. Homogenates were passed thorough a 20 μm filter, diluted (10E-1 and 10E-2), and 0.1 mL was plated on McConkey agar plates. Dishes were incubated at 37° C, and CFUs were counted manually 24 hr later.

Depletion of liver macrophages

Anesthetized rats were intravenously (penile vein) administered clodronate-encapsulated liposomes or an equal volume of phosphate buffered saline (PBS). Clodronate-encapsulated liposomes (ClodronateLiposomes.com, Haarlem, The Netherlands) were administered 24 h post an i.p. *E. coli* or vehicle administration, at a concentration of 9 μl/g body weight. The timing and dose of liposome administration was chosen based on well-established studies demonstrating maximum depletion of Kupffer cells in liver tissue (Van Rooijen and Sanders, 1996; van Rooijen et al., 1996). It should be noted that this route of administration (i.v.) selectively depletes macrophages in liver, spleen, and bone marrow, but does not target macrophages in other compartments such as lymph nodes, peritoneal cavity, testes, alveolus, or synovium.

Tissue sections and immunohistochemistry

Sections of spleen and liver were immediately frozen in liquid nitrogen following cardiac saline perfusion (as described above), and were stored in -80°C until processed. Serial sections (10μm) were cut at -20°C from tissue blocks that contained spleen or liver samples from each treatment group and thaw mounted onto gelatin-subbed slides. Slides were again stored at -80°C until processed. Slides were taken out of -80°C and dried for 10 minutes at 37°C and then fixed in cold acetone for 10 minutes. Slides were then quenched in methanol with 0.3% hydrogen peroxide for 30 minutes. To reduce non-specific reactivity due to endogenous biotin, pre-treatment with a biotin blocking kit (Vector Laboratories, Peterborough, UK) was done according to the manufacturer's instructions. Recruited monocytes and activated resident macrophages were detected using the anti-ED-1 serum (Serotec, Oxford, UK) at a concentration of 1:200. The anti-ED-2 (Serotec, Oxford, UK) serum was used also at a concentration of 1:200 to detect mature macrophages in the spleen and in the liver. Cell markers were then detected using a biotinylated goat anti-mouse secondary antibody (Jackson ImmunoResearch Labatories, West Grove, PA) at a concentration of 1:100 with standard ABC amplification. Inactive di-aminobenzamidine (DAB) solution was made using .2% DAB in 0.1M phosphate buffer with 0.0000008% glucose oxidase added after filtration and slides were incubated in the inactive DAB for 10 minutes. DAB was then activated with 0.04% β-D glucose and produced a brown insoluble precipitate that revealed immunoreactivity. Manual cell counts were accomplished by an experimenter blind to the treatement conditions using the Image J cell counter plugin tool.

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Context pre-exposure fear conditioning

A context pre-exposure fear conditioning paradigm was employed to measure memory performance, as we have previously reported this paradigm to be highly sensitive to disruptions to the hippocampus (Barrientos et al., 2002; Rudy et al., 2002; Barrientos et al., 2006). Contextual fear conditioning depends on two processes: the construction of a conjunctive representation of the conditioning context, and the association of that representation with shock. We have established that acquiring a conjunctive representation depends on an intact hippocampus. Because in this paradigm the two processes are presented independently (on separate days) it allows us to more readily detect impairments selective to the hippocampus. The conditioning context was one of two identical Igloo ice chests (54 L × 30 W × 27 H, cm) with white interiors. A speaker and an activated 24-V DC light bulb were mounted on the ceiling of each chest. The conditioning chambers (26 L × 21 W × 24 H, cm), placed inside each chest, were made of clear plastic and had window screen tops. A 2 sec, 1.5 mA shock was delivered through a removable floor of stainless steel rods 1.5 mm in diameter, spaced 1.2 cm center to center. Each rod was wired to a shock generator and scrambler (Coulbourn Instruments, Allentown, PA). Chambers were cleaned with water before each animal was conditioned or tested. Rats were taken two at a time from their home cage and transported to conditioning context in a black ice bucket with the lid on so that the rats could not see where they were being taken. Rats were placed in the context and allowed to freely explore and then were transported back to their home cage where they remained approximately 40 sec before the next exposure. This procedure was repeated 6 times. Rats remained in the novel context for 5 min on the first exposure and for 40 sec on the 5 subsequent exposures. The rats were transported in the black bucket each time that they were returned to their home cage, but with the lid off, so that they could discern whether they were headed to the context or to their home cage. The purpose of these multiple exposures was to establish the features of the black bucket as retrieval cues that could activate the representation of the context (for further detail see (Rudy et al., 2002)). Forty-eight h later, each animal was taken from its home cage and transported to the conditioning context in the black bucket. There, they received one 2 s shock immediately after being placed in the context. They were then quickly taken out of the context and transported back to their home cage. The rats' time in the conditioning context never exceeded 5 sec. To establish a baseline of generalized fear, 24 h later, freezing behavior was assessed over a 6 min period in a novel, neutral, control context. The control context was a circular animal enclosure with a solid plastic floor and ceiling, and wire walls. Attached to the wall of each enclosure was a small plastic running wheel (the size of which is suitable for a hamster or mouse, but not a rat) to serve only as a visual or manual distraction. To assess the rats' memory for the conditioning context, freezing behavior while in the conditioning context was scored over a 6 min period. The order of these two tests was counterbalanced across the groups so that half of each group was tested in the control context first, and in the conditioning context several hours later, and the other half was tested in the conditioning context first, and in the control context several hours later. After placing the rat into the respective context, every 10 s each rat was judged as either freezing or active at the instant the sample was taken. Freezing, the rat's dominant defensive fear response, is a complete suppression of behavior that is accompanied by immobility, shallow breathing, and a variety of other autonomic changes including an increase in heart rate and pilo-erection (Kim and

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Fanselow, 1992). Freezing in these experiments was defined as the absence of all visible movement, except for respiration. Scoring began approximately 10 sec after the animal was placed into the chamber. Scoring was carried out by observers blind to experimental treatment and inter-rater reliability exceeded 97% for all experiments.

This experiment was conducted with two groups of aged rats. One group received an *E. coli* infection, and the other received a saline injection, both groups then received clodronate for macrophage depletion (as described above). Control groups for the clodronate condition (that is, rats receiving saline instead of clodronate) were not included for the following rationale. Because aged F344xBN F1 rats are available on a very limited basis (recent NIA restrictions allow only 20 rats per month, per grant), sufficient animals were not available to run the control groups at the same time as the clodronate groups. Running the control groups at a different time, as a separate experiment, would have been in vain, as we have multiple papers demonstrating the cognitive effects of saline or *E. coli* (in rats that have not received clodronate) on aged rats using this same behavioral paradigm (Barrientos et al., 2006; Barrientos et al., 2009b; Frank et al., 2010b; Barrientos et al., 2011). Furthermore, these effects are very stable across experiments. To make this evident, a red dotted line was added to Fig. 5 indicating freezing percentages of vehicle and E. coli-injected aged rats from our most recent publication using this paradigm (Barrientos et al., 2011).

Statistical analyses

Statistical analyses were conducted using StatView v.5.0 and GraphPad Prism v.5.0 software. The specific statistical test used for any given experiment is specified in the results section of each experiment. In many cases a two-way 2×2 ANOVA was employed. In a few cases, where there were only two groups being compared, unpaired Student's *t*-tests were used. Where appropriate (when the interaction term was significant), Scheffe's post-hoc tests were conducted to reveal pairwise differences between groups. Statistical significance for all tests was set at alpha = 0.05.

Results

Proinflammatory response in spleen & liver of young and aged rats following E. coli infection

To determine whether liver responses are exaggerated and/or protracted in the liver of aged rats, protein levels of 3 principle proinflammatory cytokines, IL-1β, IL-6, and TNFα, were measured 4 days post saline or *E. coli* infection, in young and aged rats. Previous findings from our laboratory demonstrated that IL-1β expression in the spleen 4 days following an *E. coli* infection had returned to baseline levels (Barrientos et al., 2009a), therefore, spleen was also assayed here to confirm these previous findings. Results showed that only aged rats that had received *E. coli* 4 days earlier showed a potentiated increase in proinflammatory cytokines in liver. All other groups were indistinguishable. A multifactorial (2×2) two-way ANOVA for the three cytokines in liver produced a significant interaction term (IL-1β: ($F(1,26) = 9.42$, $P = 0.005$); IL-6: ($F(1,26) = 5.19$, $P = 0.03$); TNFα: ($F(1,26) = 10.94$, $P = 0.003$) (See Figure 1). Scheffe post hoc tests revealed that cytokine levels were significantly elevated in *E. coli*-infected aged rats compared to *E. coli*-infected young rats (IL-1β: P <

0.05; IL-6: $P < 0.05$; TNFα: $P < 0.05$), and vehicle-injected aged rats (IL-1β: $P < 0.001$; IL-6: $P < 0.05$; TNFα: $P< 0.01$). No other groups differed from each other (Fig 1a, b, c). In the spleen, we surprisingly found that IL-1β was still elevated in *E. coli*-infected aged rats, but this was not the case with IL-6 or TNFα. A two-way ANOVA for the IL-1β in spleen produced a significant interaction term ($F(1,25) = 4.32$, $P = 0.048$). Scheffe post hoc tests revealed that IL-1β was significantly elevated in *E. coli*-infected aged rats compared to *E. coli*-infected young rats ($P < 0.01$) and vehicle-injected aged rats (IL-1β: $P < 0.01$). There was no significant interaction for IL-6: ($F(1,20) = 0.91$, $P = 0.35$), or TNFα: ($F(1,26) = 0.92$, $P = 0.35$) (See Figure 1d, e, f).

Bacterial clearance in spleen & liver of young and aged rats following E. coli infection

One possible explanation for the potentiated inflammatory response observed in the liver and spleen is that perhaps bacterial clearance in these organs is impaired in aged rats. To examine this possibility, we compared bacterial clearance in the liver and the spleen in young and aged rats four days after *E. coli* inoculation. In addition, we examined bacterial numbers in hippocampus to assure that *E. coli* does not enter the brain. Results from both liver and spleen showed no differences in CFU between the age groups. Because vehicle-treated rats had no detectable bacterial numbers, a t-test was used to analyze data only between the groups that had been infected with *E. coli*. CFUs in the liver revealed no effect of age ($t=0.18$, $df=12$, $p = 0.86$) (Fig. 2a). Similar results were observed in the spleen ($t=0.63$, $df=11$, $p = 0.54$) (Fig. 2b). Zero CFUs were found in the hippocampus in all animals (Fig 2c), confirming that *E. coli* bacteria did not get into the brain in these animals. These findings indicate that the potentiated inflammatory response observed in the liver and spleen of aged rats could not be explained by a deficit in bacterial clearance.

The effects of depleting peripheral macrophages following E. coli challenge on IL-1β expression in aged rats

Previous work has demonstrated that Kupffer cells play a significant role in the magnitude of the neuroinflammatory response following an inflammatory challenge to the brain (Campbell et al., 2008). To further understand whether peripheral macrophages of aged rats drive the long-lasting pro-inflammatory response observed in the hippocampus of aged rats following a peripheral inflammatory challenge, hepatic and splenic macrophages were depleted using intravenously administered clodronate-encapsulated liposomes. Because it is well established that liver macrophages are necessary to initiate a response in the brain (Derijk et al., 1993; Campbell et al., 2008), *E. coli* was injected one day prior to clodronate, to allow IL-1β signaling in the hippocampus before macrophage depletion. Rats were euthanized 4 days following *E. coli* (3 days post-clodronate), and liver and spleen tissue samples were stained immunohistochemically for the presence of activated resident, and/or proliferating macrophages (ED-1) and mature macrophages (ED-2). Additionally, IL-1β was assayed in liver, spleen, and hippocampus. Immunohistochemical analysis of liver and splenic tissues confirmed that both mature and new macrophages were depleted in rats treated with clodronate (Fig. 3a-d). A 2×2 two-way ANOVA of ED1- and ED2-positive cells in liver samples revealed only a significant main effect of clodronate treatment (ED1: ($F(1,19) = 37.53$, $P < 0.0001$; ED2: ($F(1,17) = 15.74$, $P < 0.001$). Similarly in spleen, there was only a significant main effect of clodronate treatment (ED1:($F(1,19) = 24.76$, $P <$

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0.0001; ED2: ($F(1,18) = 10.35$, $P < 0.01$). These data confirm that clodronate treatment did indeed have the desired effect of depleting macrophages in these organs. With regard to the IL-1β response, in liver and spleen, this proinflammatory cytokine was robustly elevated following *E. coli*-treatment, and clodronate treatment dramatically reduced that response, as expected (Fig 4a & 4b). A 2 × 2 two-way ANOVA revealed a significant interaction term for both liver: ($F(1,19) = 57.72$, $P < 0.0001$), and spleen: ($F(1,19) = 8.28$, $P < 0.01$). Scheffe post hoc tests confirmed that *E. coli*+vehicle-injected rats exhibited a significant elevation in IL-1β compared to the vehicle+vehicle-injected group (liver: $P < 0.0001$; spleen: $P < 0.05$). Importantly, a dramatic reduction in IL-1β was observed in *E. coli*+clodronate-injected rats compared to *E. coli*+vehicle-injected rats (liver: $P < 0.0001$; spleen: $P < 0.0001$). In the hippocampus (Fig 4c), IL-1β was robustly elevated following *E. coli*-treatment, as we have demonstrated previously (Barrientos et al., 2009b). Remarkably however, clodronate treatment did not attenuate that proinflammatory response. A 2 × 2 two-way ANOVA confirmed a significant main effect of peripheral challenge ($F(1,18) = 32.75$, $P < 0.0001$), but no main effect of clodronate treatment ($F(1,18) = 0.11$, $P < 0.74$), and no interaction ($F(1,18) = 0.15$, $P = 0.70$). These results strongly suggest that liver and/or spleen macrophages are not necessary for the long-lasting maintenance of the hippocampal proinflammatory response.

The effects of depleting peripheral macrophages following E. coli challenge on hippocampal memory in aged rats

To further examine the role peripheral macrophages play in the neuroinflammatory-induced memory impairments observed in aged rats 4 days following an *E. coli* infection, hepatic and splenic macrophages were depleted as in the previous experiment and hippocampal memory performance on a contextual fear conditioning paradigm was measured. First, it is important to note that during the pre-exposure procedure 4 days after E. coli and 3 days after clodronate, all animals actively explored the context and did so to the same degree (Fig. 5 inset). These findings indicate that at this time point none of the animals were actively sick or lethargic due to any of the treatments. Additionally, it should be noted that these findings are typical of animals that have not received clodronate. With regard to the memory test, vehicle+clodronate rats performed well, freezing on average 52% of the time. This level of freezing is also typical of a vehicle-injected rat that has not received clodronate (as depicted with the dotted line on Fig. 5), suggesting that clodronate alone did not alter memory performance. *E. coli*+clodronate rats exhibited poor memory for the conditioning context, freezing on average 19% of the time. Again, this level of reduced freezing is typical of an *E. coli*-infected aged rat (as depicted with the dotted line on Fig. 5), suggesting that the clodronate injection did not alter the typically observed *E. coli*-induced memory performance. An unpaired t-test showed that these groups were significantly different from each other ($t=2.99$, $df=11$, $p = 0.012$) (Fig. 5). A similar analysis of freezing behavior in a control context (to control for generalized fear) produced no main effect of clodronate treatment ($t=1.22$, $df=12$, $p = 0.25$) (Fig 5). These findings further suggest that peripheral macrophages coming from liver or spleen do not play a significant role in memory deficits produced by a peripheral infection in aged rats.

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Discussion

Overall, the present study strongly suggests that age-related sensitization of hepatic and splenic macrophages are not critical mediators of the exaggerated and long-lasting neuroinflammatory response in the hippocampus of aged rats following a bacterial infection, nor of the memory impairments associated with that response. Four days after *E. coli* infection, aged rats exhibited a potentiated proinflammatory cytokine response in the liver, and to a lesser extent in the spleen, compared to young adult rats. In liver, significant elevations in IL-1β, IL-6, and TNFα were observed, whereas in spleen only IL-1β was elevated. It was surprising to find a significant elevation of IL-1β in the spleen at 4 days, as we had previously reported that IL-1β levels in spleen were not significantly elevated at 4 days compared to young infected, or vehicle control levels (Barrientos et al., 2009b). Upon revisiting these old data, a similar trend in IL-1β can be observed at this time point between the groups, however it did not reach statistical significance. It is worth noting however, that the other proinflammatory cytokines (IL-6 and TNFα) measured here were not elevated in spleen. Since cytokine levels in liver were examined at only one time point (4 days), we cannot assess how long these proinflammatory cytokines remain elevated past the 4 day time point. However, based on our previous findings that sickness behaviors (fever and reduced physical activity) in aged rats have dissipated by day 4 (Barrientos et al., 2009a), and these behaviors are known to be driven by peripheral macrophages (Derijk et al., 1993), it is likely that this response does not persist too much past this time point.

E. coli colony forming units in both the spleen and liver were not different between young and aged rats 4 days after infection, suggesting that clearance of *E. coli* bacteria, at least in these organs, is not impaired in aged rats, and thus cannot account for the protracted cytokine response measured there. The present findings are consistent with previous reports demonstrating no differences in bacterial clearance between otherwise healthy young and aged mice (Wyde et al., 1989). Conversely, bacterial clearance deficits have been reported in aged rats, but it is important to note that in these cases, the endotoxin dose was extremely high (approximately one order of magnitude or more higher than in our model), and caused 100% mortality (Horan et al., 1991).

Clodronate-encapsulated liposome treatment robustly depleted hepatic and splenic macrophages, as quantified with immunohistochemistry. We also demonstrated that macrophage depletion one day after *E. coli* inoculation robustly reduced IL-1β levels in both liver and spleen tissue at day 4 post-*E. coli*. Strikingly, though the peripheral cytokine response (from liver and spleen) was ablated, the cytokine response in the hippocampus continued to be as elevated as in animals that received *E. coli* without clodronate. Had hepatic and splenic macrophages been critical mediators of the long-lasting cytokine response in the hippocampus, depleting them should have also reduced the response in the hippocampus, and this was not the case. Therefore, these data strongly suggest that the prolonged inflammatory response in the hippocampus of aged rats following an infection is independent of the peripheral response in liver and spleen. As noted earlier, peripheral macrophages from other compartments were not depleted, thus there remains a possibility that macrophages from these other compartments could be driving the proinflammatory effect in the aged hippocampus. In addition, it should be noted that the claim is not that

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hepatic or splenic macrophages are uninvolved in the *initial* signaling to the brain, as there is abundant evidence to support this idea (Maier, 2003; Blatteis et al., 2004). In fact, macrophage depletion was purposely delayed until a day after bacterial infection, thereby allowing peripheral macrophages to initiate the central response. Thus, our data supports the claim that hepatic and splenic macrophages are not involved in the *maintenance* of the long-lasting response exhibited in the hippocampus after initial stimulation. These data are consistent with our recent finding that voluntary exercise in aged rats robustly prevented *E. coli*-induced IL-1β elevations in the hippocampus as well as the associated hippocampal-dependent memory deficits (Barrientos et al., 2011). This is consistent because exercise did not lower *E. coli*-induced IL-1β levels in liver, suggesting that proinflammatory cytokine levels in the liver are not as critical in producing, or preventing hippocampal-dependent memory deficits.

To further demonstrate that peripheral macrophages do not mediate the *E. coli*-induced memory deficits, clodronate-encapsulated liposomes were used to deplete hepatic and splenic macrophages in aged rats that had received either an injection of saline or *E. coli* a day before. Had proinflammatory peripheral macrophages been critical to impairing hippocampal-dependent memory, depleting these would have prevented the usual cognitive deficits, but it did not. Although both groups received clodronate, rats that were infected with *E. coli* displayed a significant memory deficit compared to those that only received saline. This deficit was specific to the context in which the rats were conditioned, and was not just reflective of generalized fear for instance, as freezing in a neutral, novel context was very low and equal between the two groups.

Though other possible mechanisms mediating prolonged inflammatory responses in the aged hippocampus were not examined in the current study, there is reason to believe that sensitized hippocampal microglia may play a prominent role. Microglial markers of activation are significantly elevated in the hippocampus of aged rodents (Frank et al., 2006). Furthermore, LPS-stimulated microglia isolated from aged rodents produce a potentiated proinflammatory response compared to that produced from cells isolated from young adult rodents (Frank et al, 2010; Henry et al., 2009). Moreover, treatments that reduce microglial sensitization in aged rodents normalize the central inflammatory response to an immune challenge and prevent memory impairments (Barrientos, et al., 2011).

Together, these findings suggest that although aged rats exhibit elevated proinflammatory cytokine levels in liver and spleen beyond the response exhibited in serum, this response, inasmuch as it is driven by macrophages, is not responsible for the prolonged and exaggerated proinflammatory response exhibited in the hippocampus, nor for the memory deficits associated with that response. These findings are important for informing the field of potentially useful versus useless therapeutic targets.

Acknowledgments

This work was supported by grants from the National Institute on Aging R01AG028271 to R.M.B., M.G.F., L.R.W., & S.F.M.

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Highlights

Hepatic and splenic macrophages do not play a major role in the long-lasting maintenance of the proinflammatory response in the hippocampus of aged rats following a bacterial infection, or the memory declines that this response produces.

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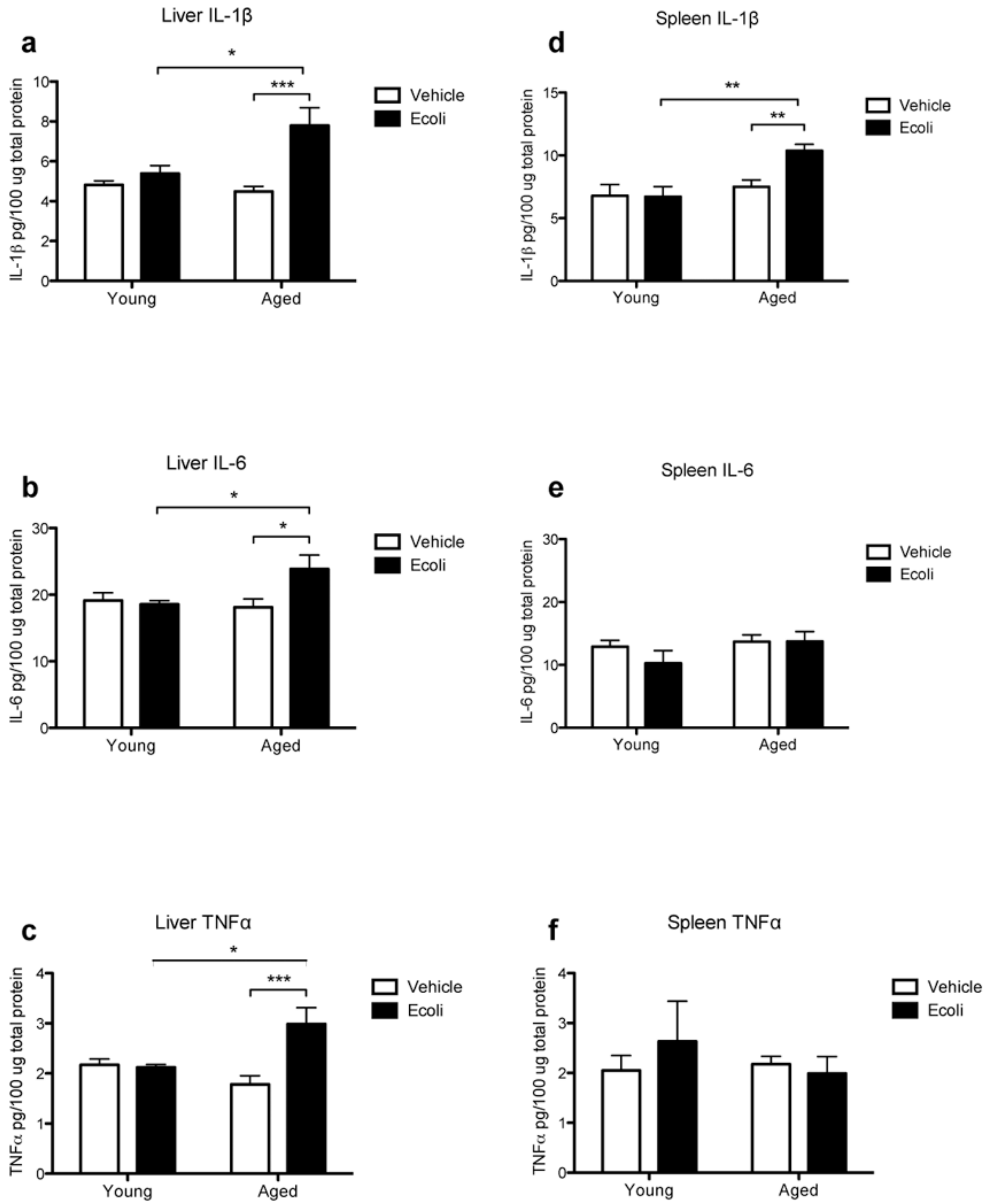

Figure 1.

Proinflammatory response in liver & spleen of young and aged rats following *E. coli* infection. Liver (a, b, c) and spleen (d, e, f,) levels of the proinflammatory cytokines IL-1β, IL-6, and TNFα, respectively, four days following either a vehicle or *E. coli* injection in young and aged rats. Error bars represent S.E.M. *p<0.05; **p<0.01; ***p<0.001.

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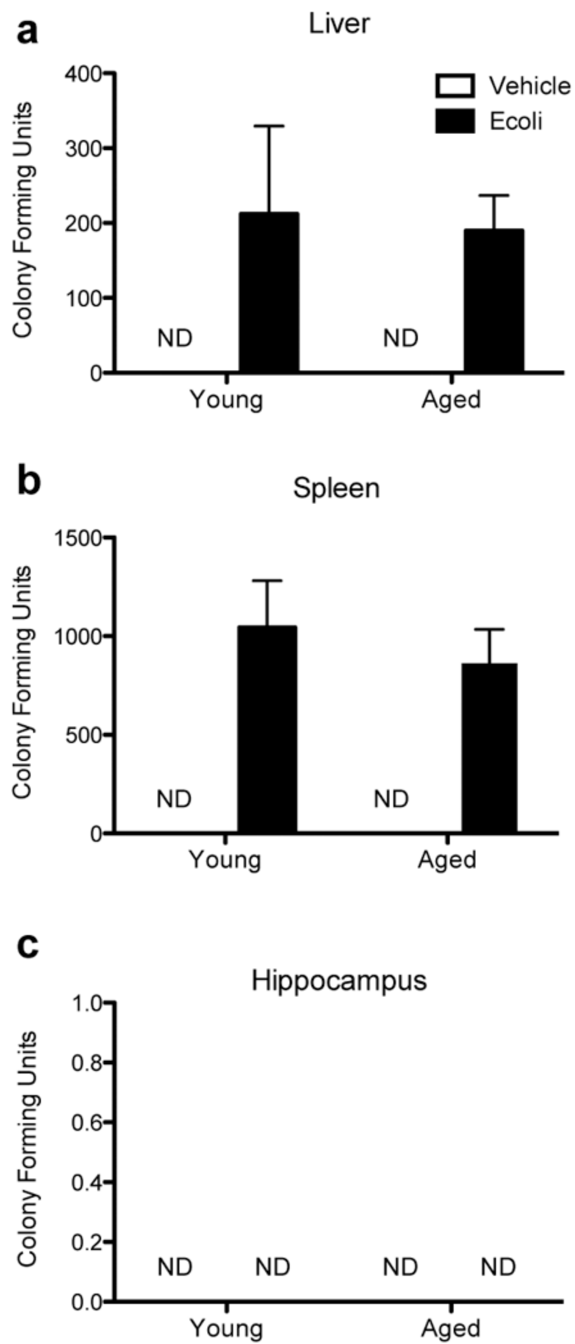

Figure 2.

E. coli colony forming units in young and aged rats four days following an injection of saline or *E. coli* in a) liver, b) spleen, and c) hippocampus. Error bars represent S.E.M. ND=not detectable.

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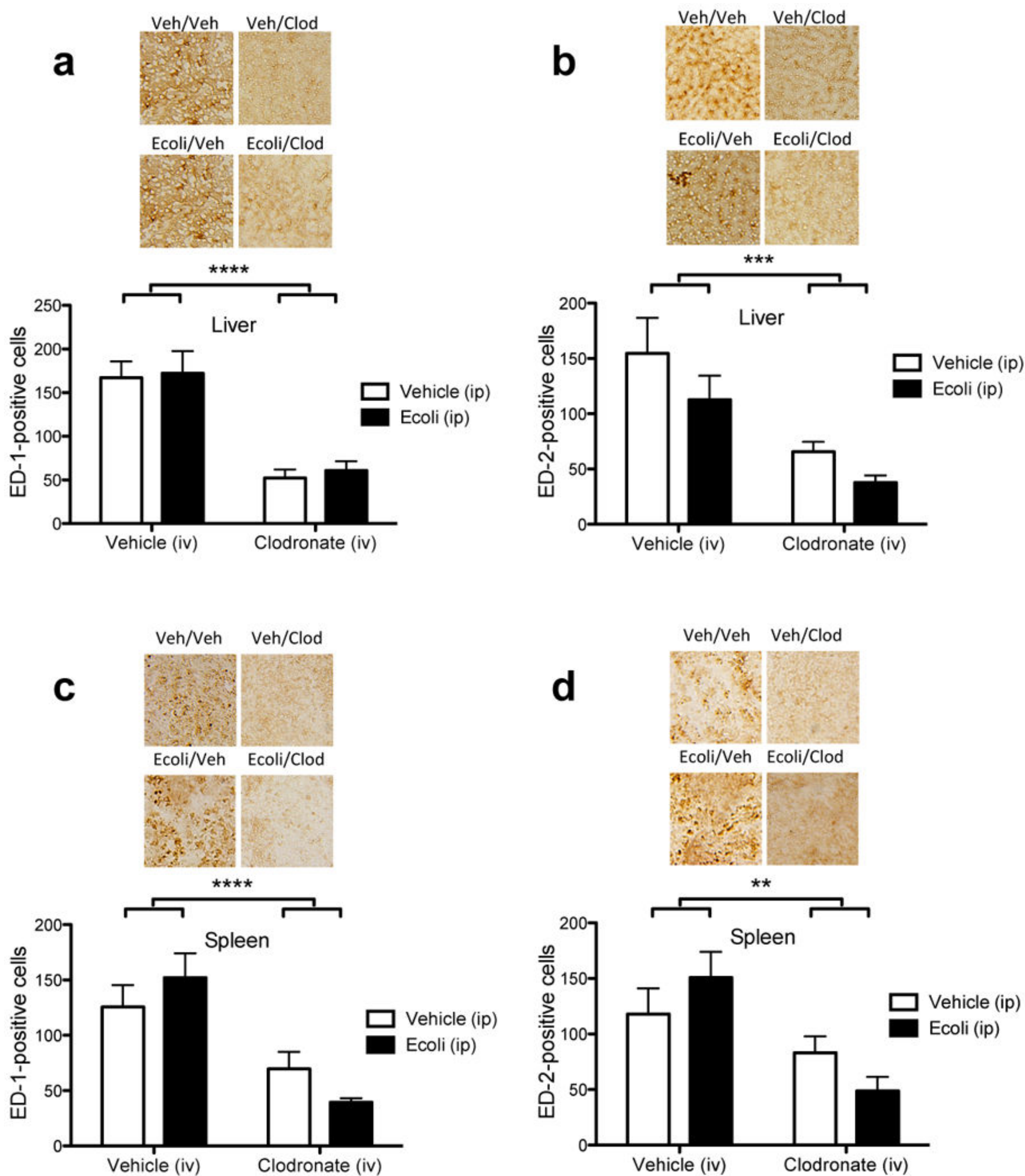

Figure 3.

ED-1 and ED-2-positive cell counts in liver (a & b) and spleen (c & d) from aged rats that had received an i.p. injection of either saline or *E. coli* followed one day later by an i.v. injection of either saline of clodronate-encapsulated liposomes. Error bars represent S.E.M. **p<0.01; ***p<0.001; ****p<0.0001.

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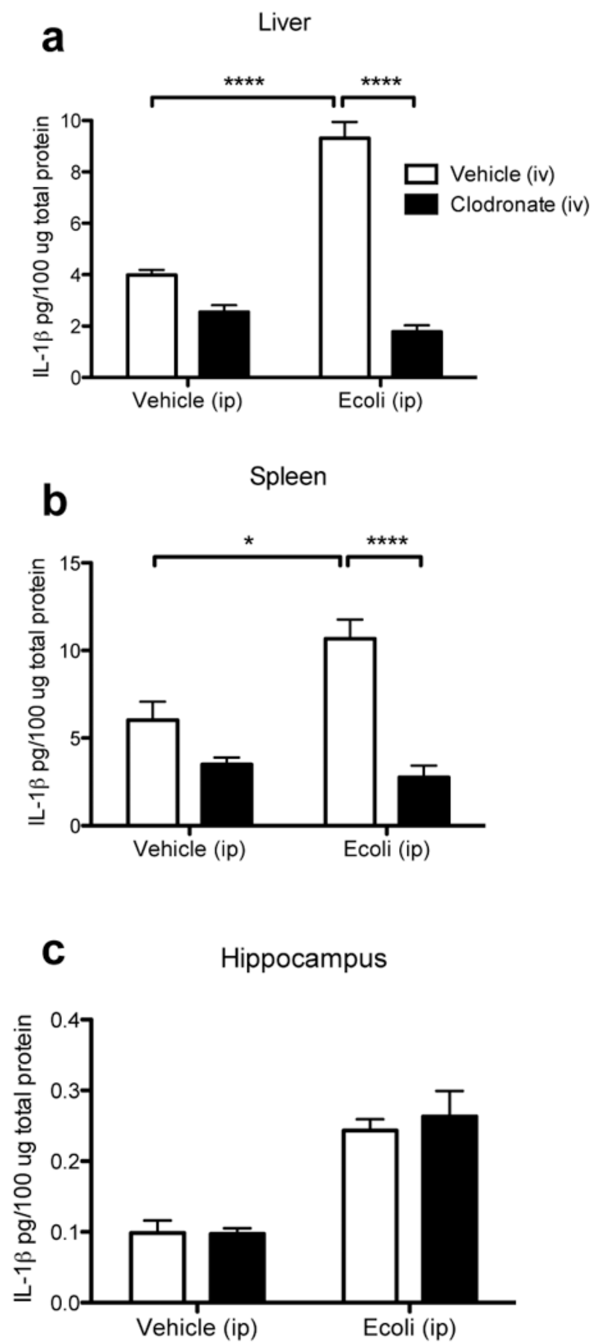

Figure 4.

IL-1β response in a) liver, b) spleen, and c) hippocampus in aged rats that had received an i.p. injection of either saline or *E. coli* followed one day later by an i.v. injection of either saline of clodronate-encapsulated liposomes. Error bars represent S.E.M. *p<0.05; ****p<0.0001.

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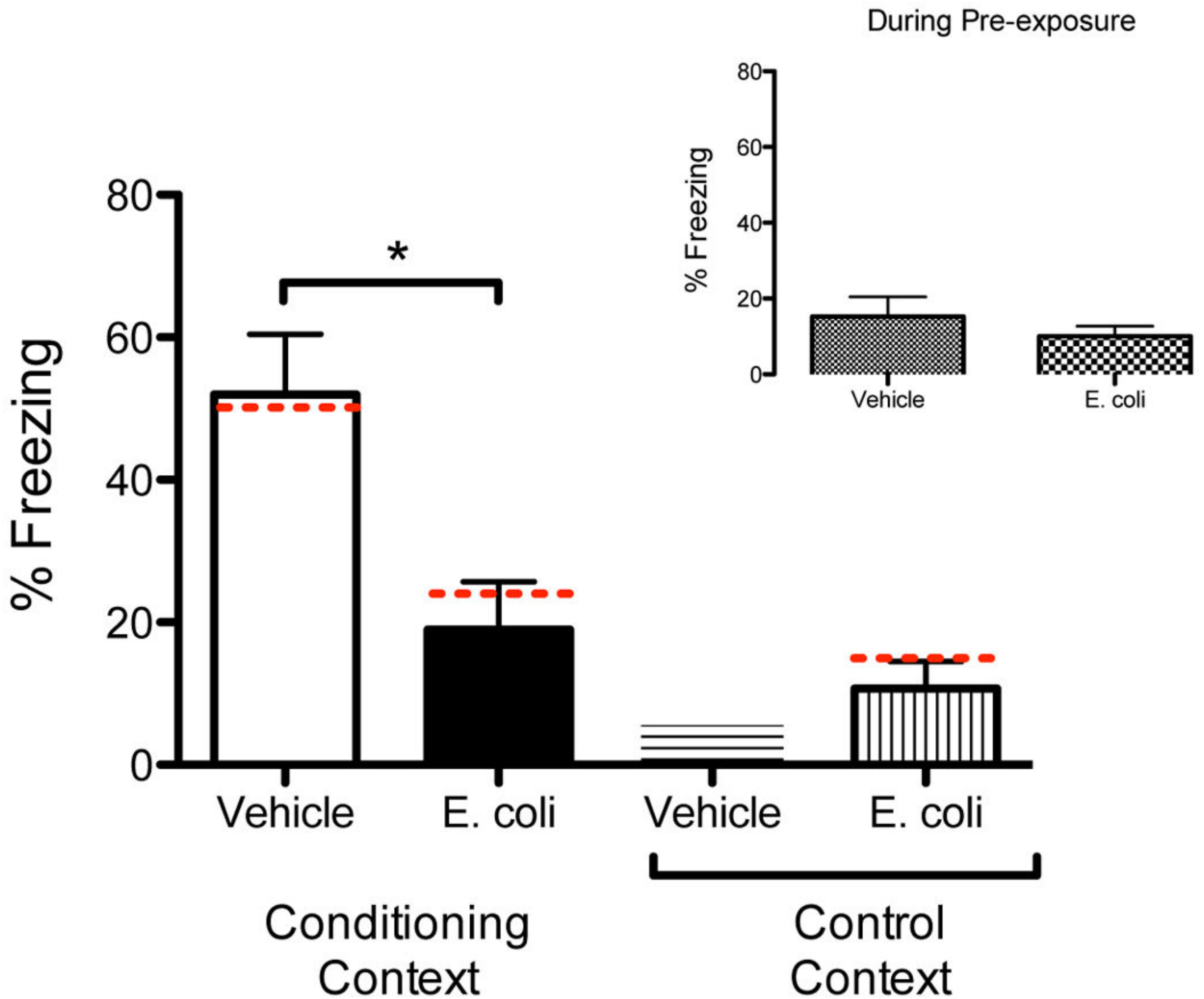

Figure 5.

Memory performance (as measured by % freezing) in aged rats, all of whom received an i.v. injection of clodronate-encapsulated liposomes one day after receiving an i.p. injection of saline or *E. coli*. Left-hand panel: Hippocampal-dependent memory as measured by % freezing upon exposure to the conditioning context. Right-hand panel: Generalized fear as measured by % freezing upon exposure to a novel, control context. Dotted lines represent vehicle- and E. coli-injected levels of freezing in rats that have not received clodronate (taken from Barrientos et al., 2011). Inset: % freezing during the pre-exposure procedure, as a measure of active exploration. Error bars represent S.E.M. *$p<0.05$