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The role of hepatic and 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 *Escherichia 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.

1. 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., 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 *Escherichia coli* infection in young and aged rats (Barrientos et al., 2009b). 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

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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, 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.

2. Materials and methods

2.1. 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 months old and weighed approximately 550 g. Young adult rats were 3 months 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.

2.2. 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 *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 h) 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).

2.3. Tissue dissection and detection of proinflammatory cytokines

Four days following the *E. coli* or vehicle injection, animals were anesthetized with 50 mg/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 s using an ultrasonic cell disrupter (Fisher Scientific, Pittsburgh, PA), centrifuged at 10,000*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 μg of total protein.

2.4. 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 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 50 mg/kg of sodium pentobarbital. Rats were then transcardially perfused with cold 0.9% saline. Spleens (1 g), livers (1 g), and one hemisphere of hippocampus were dissected out, and homogenized in 3 mL PBS with a hand-held glass homogenizer. Homogenates were passed through a 20 μm filter, diluted (10E-1 and 10E-2), and 0.l mL was plated on McConkey agar plates. Dishes were incubated at 37 °C, and CFUs were counted manually 24 h later.

2.5. 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.

2.6. 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 (10 μm) 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 min at 37 °C and then fixed in cold acetone for 10 min. Slides were then quenched in methanol with 0.3% hydrogen peroxide for 30 min. 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