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Dysfunction of Inflammation-Resolving Pathways Is Associated with Exaggerated Postoperative Cognitive Decline in a Rat Model of the Metabolic Syndrome

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The cholinergic antiinflammatory pathway (CAP), which terminates in the spleen, attenuates postoperative cognitive decline (PCD) in rodents. Surgical patients with metabolic syndrome exhibit exaggerated and persistent PCD that is reproduced in postoperative rats selectively bred for easy fatigability and that contain all features of metabolic syndrome (low-capacity runners (LCRs)). We compared the CAP and lipoxin A_4 (LXA_4), another inflammation-resolving pathway in LCR, with its counterpart high-capacity runner (HCR) rats. Isoflurane-anesthetized LCR and HCR rats either underwent aseptic trauma involving tibial fracture (surgery) or not (sham). At postoperative d 3 (POD3), compared with HCR, LCR rats exhibited significantly exaggerated PCD (trace fear conditioning freezing time 43% versus 57%). Separate cohorts were killed at POD3 to collect plasma for LXA4 and to isolate splenic mononuclear cells (MNCs) to analyze CAP signaling, regulatory T cells (Tregs) and M2 macrophages (M2 Mϕ). Under lipopolysaccharide (LPS) stimulation, tumor necrosis factor (TNF)-α produced by splenic MNCs was 117% higher in LCR sham and 52% higher in LCR surgery compared with HCR sham and surgery rats; LPS-stimulated TNF-α production could not be inhibited by an α7 nicotinic acetylcholine receptor agonist, whereas inhibition by the β_2 adrenergic agonist, salmeterol, was significantly less (–35%) than that obtained in HCR rats. Compared to HCR, sham and surgery LCR rats had reduced β_2 adrenergic receptor–expressing T lymphocytes (59%, 44%), Tregs (47%, 54%) and M2 Mϕ (45%, 39%); surgical LCR rats' hippocampal M2 Mϕ was 66% reduced, and plasma LXA4 was decreased by 120%. Rats with the metabolic syndrome have ineffective inflammation-resolving mechanisms that represent plausible reasons for the exaggerated and persistent PCD.

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INTRODUCTION

Exaggerated and/or persistent postoperative cognitive decline (PCD) results in withdrawal from the labor force, dependence on others for activities of daily living and a higher mortality rate (1,2). Clinical studies to identify possible mechanisms are beset by diagnostic problems due to variability of neuropsychological tests as well as the difficulty in establishing the individual patient's baseline age-dependent trajectory of cognitive decline before the surgical intervention (3). To address this conundrum, we resorted to rodent models to determine the mechanisms underlying PCD and have defined an inflammatory cascade that is depicted in Figure 1 (4–11).

The postoperative inflammatory cascade and the resulting cognitive changes are typically short-lived because of inflammation-resolving processes that include a cholinergic reflex identified and elaborated by Tracey's laboratory (12,13). Vagal outflow ramifies in the celiac ganglion giving rise to the postganglionic splenic nerve that terminates in the spleen, where it signals via an α7 nicotinic acetylcholine receptor (nAChR) to reduce synthesis of proinflammatory

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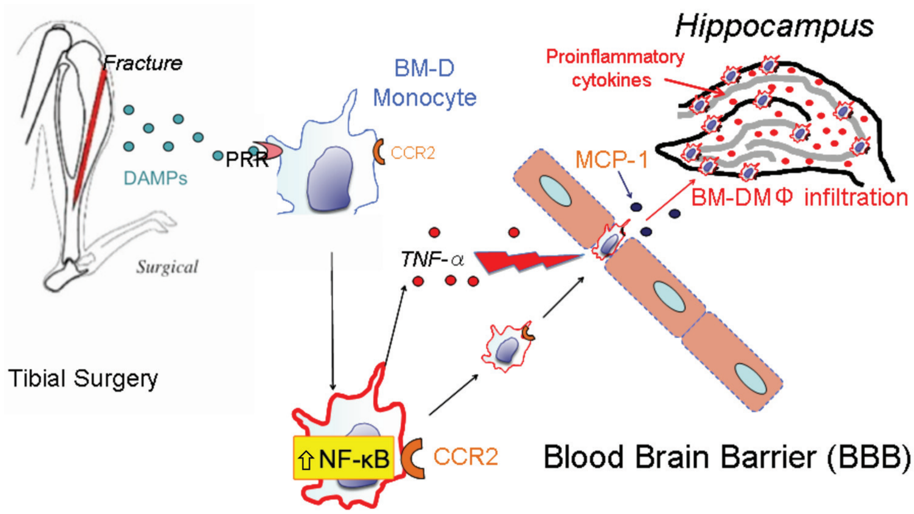

Figure 1. Postoperative neuroinflammatory response to peripheral aseptic trauma. Aseptic surgical trauma induces binding of damage-associated molecular patterns (DAMPs) to pattern recognition receptors (PRR); this engages the innate immune system via NF-κB–dependent signaling in monocytes, to synthesize and release proinflammatory cytokines, including TNF-α, which disrupts the blood brain barrier (BBB) (6–8). Through a permeable BBB, CCR2-expressing bone marrow–derived macrophages (BM-DM) are attracted by the newly expressed chemokine MCP-1 into the brain parenchyma (5). Within the hippocampus, the activated macrophages release proinflammatory cytokines that are capable of disrupting long-term potentiation, the neurobiologic correlate of learning and memory (4,9–11).

cytokines by inhibiting nuclear factor-κB (NF-κB) activity (Figure 2) (12–15). Recently, we showed the importance of this reflex for resolving aseptic trauma-induced neuroinflammation and cognitive decline. Stimulating the α7 nAChR in macrophages inhibited NF-κB activity and prevented postoperative monocyte migration into the hippocampus and cognitive decline; blockade of this signaling pathway exaggerated neuroinflammation and cognitive decline (4).

In the presence of the metabolic syndrome, comprising insulin resistance (hyperglycemia that can progress to type 2 diabetes mellitus), visceral obesity, hypertension and dyslipidemia, the risk of developing exaggerated cognitive decline is significantly enhanced (16,17); the mechanisms underlying this enhanced susceptibility are not known. To further our understanding of the processes underlying enhanced PCD in this pathological setting, we resorted to an animal model of metabolic syndrome. Since 1997, Koch and Britton (18) have selectively bred low-capacity runner (LCR) and high-capacity runner (HCR) rats. The selection criterion was the ability to perform a maximal run on a motorized treadmill at 12 wks of age (18); after 30 generations, there was a sevenfold difference in running capacity between LCR and HCR rats. The LCRs contain each of the features of the metabolic syndrome, including elevated low-density lipoproteins (LDLs), cholesterol, blood pressure, triglycerides, fasting glucose, insulin and C-reactive protein (19). We recently reported that LCR rats exhibit both exaggerated acute cognitive decline together with learning and memory defects that persist for at least 5 months after surgery when compared with HCR rats (20).

Because of the dependence on inflammation-resolving mechanisms for terminating transient PCD, we decided to explore whether defects in these pathways could provide a plausible explanation for exaggerated and persistent PCD in the LCR rats. Therefore, we investigated (a) whether splenic inflammatory cells from LCR rats are less responsive to cholinergic- and adrenergic-mediated resolution of inflammation induced by LPS and surgical challenges; (b) whether LCR rats have defects in the balance of M1/M2 macrophages, regulatory T cells and β_2 adrenergic receptor (β_2 AR)-expressing, acetylcholine-synthesizing T lymphocytes in the spleen; and (c) whether eicosanoids involved in inflammation resolution are appropriately biotransformed. The findings from this study provide new molecular targets for diagnostic and therapeutic strategies to both identify high-risk elective surgical patients with metabolic syndrome as well as provide opportunities to limit their cognitive decline, respectively.

MATERIALS AND METHODS

Chemicals

Nicotine, methyllycaconitine (MLA), salmeterol and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). PHA 568487, a selective agonist of α7 nAChR was purchased from Tocris Bioscience (Ellisville, MO, USA). They were all dissolved in 0.9% saline before each experiment.

Animals

The development of rats selected to be either LCR or HCR is described in detail elsewhere (18,21). In the present investigation, male HCR and LCR rats (generation 30) were housed under standard temperature and humidity laboratory conditions in which the light and dark cycles were 12 h each. Rats were tested for running capacity at the University of Michigan at 11 wks of age and shipped to the University of California, San Francisco, at 16 wks of age. The Committee on Animal Research of the University of California, San Francisco, approved all the protocols.

Aseptic Surgery of Tibia

As previously described (4), animals were anesthetized with 2.1% isoflurane in 30% FiO_2. Under full aseptic conditions, surgical animals underwent an open tibia fracture of the left hind paw with an intramedullary fixation. The left hind limb of surgical animals was meticulously shaved and disinfected with povidone iodine. Briefly, a middle incision was performed on the left hind paw, followed by the insertion of a 20-G pin in the intramedullary canal; the periosteum was then stripped and osteotomy was performed. After producing the fracture, the wound was irrigated and the skin was sutured with 8/0 Prolene sutures. Temperature was monitored and maintained optimal with the aid of warming pads (Harvard Apparatus, Holliston, MA, USA) and temperature-controlled light. Analgesia (0.1 mg/kg buprenorphine) was given subcutaneously after anesthetic induction and before skin incision. Sham animals were exposed to isoflurane anesthesia (2.1% isoflurane in 30% FiO_2) for 20 min in a temperature-controlled anesthetic chamber, shaved and received buprenorphine as described above.

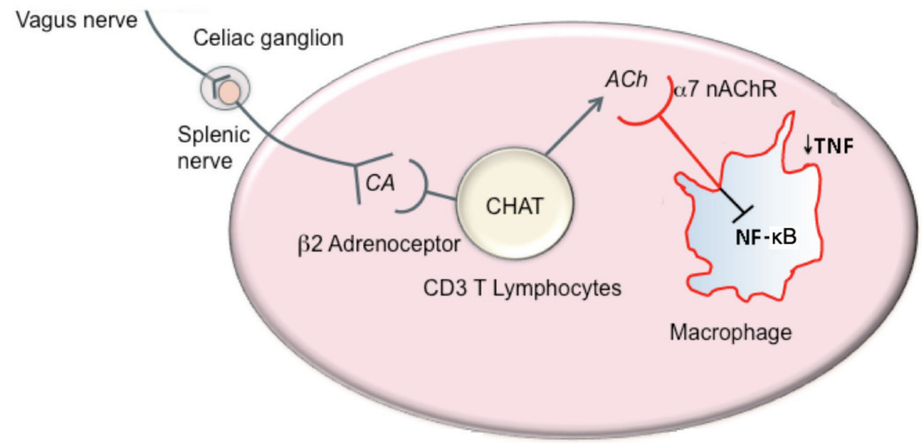

Figure 2. Cholinergic inflammation-resolving pathway (12–15). At its splenic nerve terminus, vagal outflow releases adrenergic agonists (rather than the usual cholinergic neurotransmitter); these catecholamines (CA) activate β_2 ARs on CD3 T lymphocytes that contain choline acetyltransferase (CHAT), which is capable of synthesizing the acetylcholine needed to mediate inhibition of macrophage NF-κB activity by signaling through α7 nAChR.

Isolation of Mononuclear Cells from Spleen and Peripheral Blood

Spleens were harvested, homogenized and filtered over a 70-μm nylon cell strainer (BD, Franklin Lakes, NJ, USA). The cell pellets were resuspended in 2% fetal calf serum (FCS) Dulbecco's modified Eagle medium, and then 4 mL solution was delicately layered on 3 mL Ficoll (GE Healthcare, Pittsburgh, PA, USA). For isolation of peripheral blood mononuclear cells, EDTA anticoagulated blood was diluted 1:2 with Ca^{2+} Mg^{2+}-free Hanks balanced salt solution (HBSS) (Gibco; Life Technologies, Carlsbad, CA, USA) and then delicately layered on Ficoll (GE Healthcare). Samples were centrifuged for 25 min at 700*g* and 22°C without applying a brake. The mononuclear cell interface was carefully removed by pipetting and washed twice with HBSS by stepwise centrifugation for 15 min at 300*g* and for 10 min at 90*g* for platelet removal.

Measurement of NF-κB Activity in the Splenic Mononuclear Cells

Splenic mononuclear cells (MNCs) (10^6/well) were pretreated with vehicle, PHA 568487 (1 μmol/L), MLA (1 μmol/L) or PHA 568487 (1 μmol/L) + MLA (1 μmol/L) and then stimulated with LPS (1 μmol/L). Cells were harvested 1 h later, and nuclei were extracted by using a nuclear extract kit (Active Motif, Carlsbad, CA, USA). Measurement of p65 NF-κB in the nuclear extract was performed according to the manufacturer's instructions (Active Motif). The levels of p65 NF-κB were expressed by percentage change ([value under LPS stimulation – value under phosphate-buffered saline {PBS} stimulation]/value under PBS stimulation × 100%).

Enzyme-Linked Immunosorbent Assay (ELISA) Measurements for TNF-α and Interleukin-10

The culture media of the PBS- or LPS-stimulated splenic mononuclear cells was collected at designated time points. TNF-α and interleukin (IL)-10 were measured by using ELISA kits from R&D Systems (Minneapolis, MN, USA). The levels of TNF-α and IL-10 were expressed as percentage change ([value under LPS stimulation – value under PBS stimulation])/value under PBS stimulation × 100%).

Quantitative Polymerase Chain Reaction Measurements for Hippocampal IL-6

Rats were perfused with PBS for 5 min before sample collection to avoid blood contamination. The hippocampus of the rats was rapidly collected 3 d after surgery and placed in RNAlater™ solution (Qiagen, Valencia, CA, USA). Total RNA was extracted by using an RNeasy Lipid Tissue Kit (Qiagen) treated with recombinant DNase I by using an RNase-Free Dnase Set™ (Qiagen) and reverse-transcribed to complementary deoxyribonucleic acid with a high-capacity RNA-to-cDNA Kit (Applied Biosystems; Life Technologies). TaqMan Fast Advanced Master Mix (Applied Biosystems; Life Technologies) and gene-specific primers and probes used for quantitative polymerase chain reaction (qPCR) are follows: β-actin (*ACTB*, Rn00667869_m1) and IL-6 (Rn01410330_m1). qPCR was performed by using the ABI Prism 7000 Sequence Detection System (Applied Biosystems; Life Technologies). The run method was as follows: PCR activation at 95°C for 20 s was followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. Each RNA sample was run in triplicate, and relative gene expression was calculated by using the comparative threshold cycle ΔCT and normalized to *ACTB*. Results are expressed as fold-increases relative to the HCR sham group.

Enzyme Immunoassay for Plasma Lipoxin A_4 and Leukotriene B_4

Plasma lipoxin A_4 (LXA_4) and leukotriene B_4 (LTB_4) were measured by using LXA_4 and LTB_4 enzyme immunoassay following the manufacturer's instructions (Oxford Biochemical Research, Rochester Hills, MI, USA).

Measurement of Cyclic Adenosine Monophosphate in the Cell Lysate of Splenic MNCs

A cyclic adenosine monophosphate (cAMP) enzyme immunoassay (EIA) kit was purchased from Cayman Chemical (Ann Arbor, MI, USA) and used for cAMP measurement. The change of cAMP was expressed by percentage change: ([value under LPS stimulation – value under PBS stimulation]/value under PBS stimulation × 100%).

Flow Cytometry

To analyze the β_2 AR expression on CD3 T lymphocytes, the splenocytes were washed with PBS (2.5% FCS), fixed in 2% paraformaldehyde, permeabilized with 0.2% Triton and then labeled with PE mouse anti-rat CD3 (eBioscience, San Diego, CA, USA), anti-α_2 adrenergic receptor antibody (Abcam, Cambridge, MA, USA) and isotype control antibodies. For detecting regulatory T cells, the splenocytes were labeled with fluorescent-labeled anti-CD4, -CD25, -FoxP3, and -isotype antibodies (eBioscience) after permeabilization. To examine α7 $nAChR^+CD11b^+$ cells in the spleens, the splenocytes were labeled with fluorescent-labeled anti-α7 nAChR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD11b/c antibody (eBioscience). All the samples were pretreated with Fc receptor blocking reagent to prevent nonspecific binding.

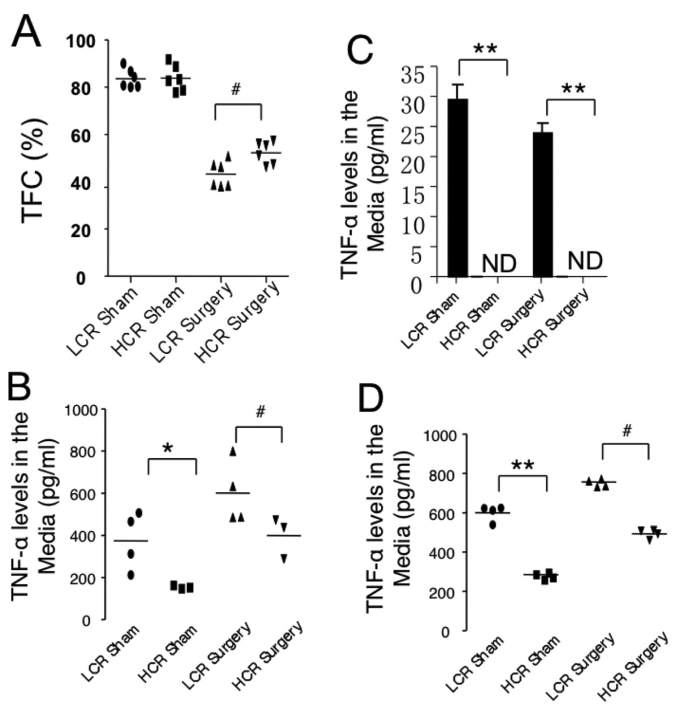

Figure 3. Postoperative cognitive decline is exaggerated in LCR rats coincident with time when splenic and peripheral MNCs in the LCR rats are more proinflammatory. (A) Freezing percentage in trace fear conditioning in LCR and HCR rats under sham and surgical conditions at d 3; $^{\#}p < 0.05$ HCR surgery versus LCR surgery. (B) TNF-α levels were higher in the media of cultured LCR splenic mononuclear cells without LPS challenge; $^{**}p < 0.01$ HCR versus LCR. (C) TNF-α levels were reduced in the media of cultured HCR blood mononuclear cells stimulated with LPS (1 μmol/L); $^{*}p < 0.05$ HCR sham versus LCR sham; $^{\#}p < 0.05$ HCR surgery versus LCR surgery at d 3. (D) TNF-α levels were decreased in the media of cultured HCR splenic mononuclear cells stimulated with LPS; $^{**}p < 0.01$ HCR sham versus LCR sham; $^{\#}p < 0.05$ HCR surgery versus LCR surgery at d 3. Each data point represents one independent experiment. Values are means ± SD. TFC, trace fear conditioning.

Fluorescent cells were analyzed with BD™ LSR Flow Cytometer (BD). Data were analyzed by FlowJo software (Tree Star Inc., Ashland, OR, USA).

Immunofluorescence for Detecting M2 Macrophages in the Hippocampus

The hemibrain was fixed in 4% paraformaldehyde in 0.1 mol/L PBS; cryoprotected in 0.1 mol/L PBS solution containing 15% sucrose (Sigma-Aldrich) for 24 h and then 30% sucrose for a further 48 h; and then sectioned and stained with anti-rat anti-CD163 (macrophage marker) and arginase 1 (M2 macrophages marker). The corresponding secondary antibodies were coupled to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes; Life Technologies) for staining. In addition, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The slides were imaged under laser-scanning confocal microscopy, and the CD163 arginase 1–positive double cells were counted in three immunolabeled sections.

Trace Fear Conditioning

As previously described (4), the behavioral study was conducted by using a dedicated trace fear conditioning chamber (Med Associates). Fear conditioning is used to assess learning and memory in rodents, which are trained to associate a conditional stimulus, such as a tone, with an aversive, unconditional stimulus, such as a foot shock. Training of trace fear conditioning consisted of placing the rats in the conditioning chamber and allowing exploration of the context for 100 s. The rats were then presented with an auditory cue (75–80 dB, 5 kHz, conditional stimulus) for 20 s. The unconditional stimulus, a 2-s foot shock (0.75 mA), was administered 20 s after termination of the tone. Rats were removed from the chamber after an additional 30 s. Freezing (the absence of all movement except for respiration) is an innate defensive fear response in rodents and a reliable measure of learned fear that can be recalled when placed in the same context on a subsequent occasion. Rats were assessed for freezing behavior on postoperative d 3.

Data Analysis

Graphpad Prism 5 (GraphPad Software) was used for statistical analysis, and results are presented as means ± standard deviation (SD). Multiple group means were analyzed by one-way analysis of variance, followed by Newman-Keuls *post hoc* test wherever appropriate. p values <0.05 were considered significant.

RESULTS

Divergence in PCD Between LCR Versus HCR Rats

Earlier, we reported that at postoperative d 7, cognitive decline is more severe in LCR versus HCR rats (20). Now we show that the percentage of freezing was significantly reduced in LCR rats compared with HCR rats in the surgical groups at postoperative d 3 (Figure 3A), suggesting that the underlying processes for the initiation and resolution of inflammation may be abnormal at this early stage.

MNCs from LCR Rat Spleen and Peripheral Blood Were More Proinflammatory than MNCs from HCR Rats

Without LPS stimulation, TNF-α in the medium of cultured splenic MNCs was higher in the LCR cells than that in the HCR cells. Levels of TNF-α did not differ between LCR sham and surgery (Figure 3B); this is unsurprising, since the postoperative rise and fall in TNF-α is completed by 24 h (6,22). After stimulation with LPS, both LCR peripheral blood and spleen MNCs produced significantly more TNF-α compared with HCR rat spleen MNCs in both sham and surgery groups (Figures 3C, D), indicating that surgery in LCR rats primes proinflammatory signaling and that TNF-α production in postoperative d 3 LCR splenic MNCs can be boosted by inflammatory (LPS) challenge. Recently, we reported that PCD is critically dependent on bone marrow–derived macrophages (5); therefore, it is noteworthy that the more severe decline of postoperative cognitive memory in LCR rats (Figure 3A) occurs at a time that bone marrow–derived macrophages were more proinflammatory (Figures 3B–D).

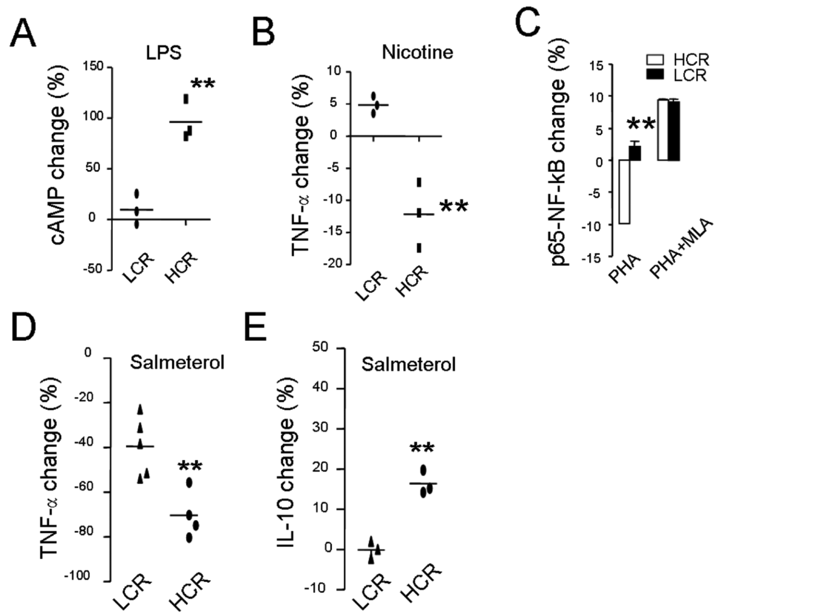

Figure 4. In response to LPS and surgery challenges, impairment of the cholinergic α7 nAChR pathway in LCR rats enhanced proinflammatory responses in splenic MNCs compared with HCR rats. (A) Decreased cAMP levels in LCR splenic MNCs stimulated with LPS (1 μmol/L); $^{**}p < 0.01$ HCR compared with LCR. (B) Less inhibitory effects of nicotine on TNF-α levels in LCR splenic MNCs stimulated with LPS; $^{**}p < 0.01$ HCR compared with LCR. (C) Less inhibitory effects of PHA 568487 (PHA, 1 μmol/L) on p65 NF-κB levels in LCR splenic MNCs stimulated with LPS; $^{**}p < 0.01$ HCR compared with LCR in PHA 568487 pretreated group; n = 3 in each group; MLA (1 μmol/L) counteracted the inhibitory effects of PHA 568487. (D) Less inhibitory effects of salmeterol on TNF-α levels in LCR splenic MNCs stimulated with LPS; $^{**}p < 0.01$ HCR compared with LCR in the salmeterol pretreated group. (E) Less effect of salmeterol (1 μmol/L) on increasing IL-10 levels in LCR MNCs stimulated with LPS; $^{**}p < 0.01$ HCR compared with LCR in the salmeterol pretreated group. Each data point represents one independent experiment. Values are means ± SD.

Dysfunction of Cholinergic Antiinflammatory Pathway in the LCR Rats

cAMP, a second messenger required for acetylcholine-α7 nAChR signaling in the splenic MNCs (23,24), can be stimulated by LPS (1 μmol/L). The cAMP levels in cell lysates were increased in HCR splenic MNCs (Figure 4A) compared with LCR MNCs, suggesting that there is a defect of cAMP production in the LCR splenic MNCs that could result in less α7 nAChR signaling.

Tracey’s group drew attention to the importance of a vagal-mediated anti-

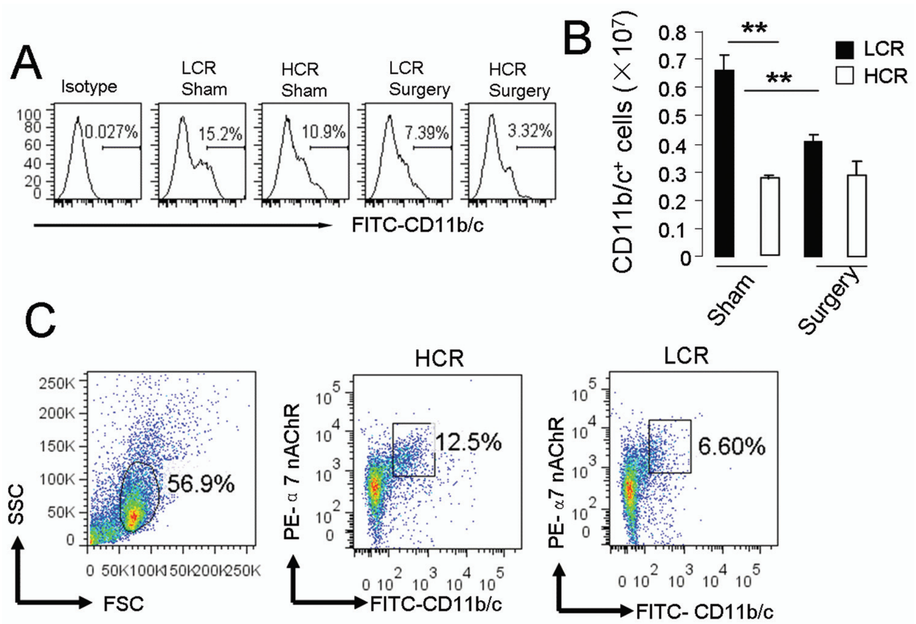

Figure 5. M1 macrophages were elevated and proresolving α7 nAChR$^+$CD11b/c$^+$ cells were decreased in the spleen of LCR rats. (A) CD11b/c was used as a marker to analyze M1 macrophages splenocytes by flow cytometry in LCR and HCR rats under sham and surgical conditions at d 3. (B) Absolute number of M1 macrophages in each spleen was calculated by multiplying the percentage of CD11b/c$^+$ cells by total number of splenocytes at d 3; n = 3 in each group. **p = 0.01 versus LCR sham. (C) Flow cytometry was used to analyze α7 nAChR$^+$CD11b/c$^+$ cells in the splenocytes. Values are means ± SD.

inflammatory reflex (12–15). Therefore, we tested whether α7 nAChR signaling is capable of inhibiting LPS-induced TNF-α production in the splenic MNCs. Nicotine, 1 μmol/L, a nonselective α7 nAChR agonist, inhibited TNF-α in the HCR splenic MNCs stimulated with LPS, but failed to affect TNF-α production in the LCR cells (Figure 4B).

To corroborate this defect in cholinergic-mediated inflammation resolution, we next pretreated isolated splenic MNCs with PHA 568487 (1 μmol/L), a selective α7 nAChR agonist (4,25) to test whether activation of α7 nAChR could reduce NF-κB activation when challenged with LPS for 1 h. PHA 568487 blocked NF-κB p65 subunit nuclear translocation in the HCR splenic MNCs stimulated with LPS; conversely, NF-κB p65 subunit nuclear translocation was not reduced in the LCR splenic MNCs, suggesting that there is dysfunction in the α7 nAChR signaling pathway. To establish the specificity of this response to the α7 nAChR, we confirmed that the α7 nAChR antagonist, MLA, blocked the PHA 568487 effect on p65 NF-κB nuclear translocation (Figure 4C).

Agonists of β_2 AR promote splenic choline acetyltransferase–expressing lymphocytes to synthesize acetylcholine (13), which is needed to inhibit NF-κB activity and the downstream synthesis and release of proinflammatory cytokines, including TNF-α. To assess this pathway, isolated splenocytes were pretreated with the selective β_2 AR agonist salmeterol (1 μmol/L) and then stimulated with LPS for 4 h to release TNF-α into the medium; salmeterol more effectively inhibited TNF-α release from splenic MNCs in HCR compared with the LCR rats (Figure 4D).

IL-10 plays an important role in down-regulating proinflammatory responses. IL-10 derived from regulatory T cells also induces a phenotypic switch from classically activated M1 (“proinflammatory”) to alternatively activated M2 (“anti-inflammatory”) macrophages (26). To test whether activation of β_2 AR facilitates IL-10 production, isolated splenocytes were pretreated with salmeterol and then stimulated with LPS for 4 h, and IL-10 was measured in the culture medium. Salmeterol more efficiently increased IL-10 production in HCR rat splenocytes than in LCR rat splenocytes (Figure 4E).

M1 Macrophages Are Increased in the Spleens of LCR Rats

M1 macrophages (CD11b/c$^+$ cells) are the major source of proinflammatory cytokines in the spleen. The number of M1 macrophages was measured by flow cytometry and whole cell population gating under both basal and surgical conditions in populations of splenocytes isolated on postoperative d 3. Under both basal and postoperative conditions, CD11b/c$^+$ cells comprised a larger percentage of splenocytes from LCR rats compared with HCR rats (Figure 5A). By multiplying the percentage of CD11b/c$^+$ cells by the total number of splenocytes, we calculated the absolute M1 macrophage number, which was significantly greater in the LCR sham group than in the HCR sham group. Absolute M1 macrophages were also deceased in the LCR surgical group compared with LCR sham, possibly because these cells were mobilized in response to surgical stress (Figure 5B). Because it is the α7 nAChR–expressing M1 macrophages (CD11b/c$^+$) that are responsive to the acetylcholine released by the vagal reflex, we considered whether reduced expression of α7 nAChR in the M1 macrophages may be responsible for the attenuation of the antiinflammatory effects of the cholinergic α7 nAChR pathway in LCR rats (Figures 5B, C). To test this, we labeled splenocytes isolated from LCR and HCR rats with both anti-α7 nAChR and CD11b/c antibodies. The whole cell population was gated during flow cytometry analysis. The α7 nAChR$^+$CD11b/c$^+$ population of splenocytes was reduced in the LCR rats (Figure 5C), suggesting that a relative reduction of acetylcholine-responding macrophages in the LCR rats may be contributing to the enhanced proinflammatory responses.

β_2 AR–Expressing CD3 Lymphocytes Are Reduced in the LCR Spleen

β_2 AR–expressing $CD3^+$ lymphocytes are acetylcholine-synthesizing cells in the spleen that are required for relaying the vagally mediated antiinflammatory signal (13). Because salmeterol had less inhibitory effect on TNF-α (Figure 4D), we explored whether there is a reduction in the population of β_2 AR–expressing lymphocytes ($CD3^+$) in LCR rat splenocytes by using anti-rat CD3 and β_2 AR antibodies and flow cytometry. β_2 AR^+CD3^+ lymphocytes were reduced in both LCR sham and surgery groups (Figure 6A), which suggest that the reduced β_2 AR^+CD3^+ lymphocyte population may contribute to less attenuation of inflammation through the α7 nAChR pathway (Figures 4D, E).

Regulatory T Cells ($CD4^+CD25^+FoxP3^+$) Are Reduced in the LCR Rat Splenocytes

It was reported that the antiinflammatory function of vagal nerve activity also depends on regulatory T cells (27) and that the α7 nAChR plays a critical role in regulating immunosuppressive function of $CD4^+CD25^+$ Tregs (28). Tregs are also required for polarizing macrophages into the alternatively activated M2 phenotype (29). Therefore, we next tested whether regulatory T cells (Tregs) are reduced in LCR rat spleen. Splenocytes were isolated from both LCR and HCR rats in sham and surgery groups. Cells were labeled with anti-rat CD4, CD25 and FoxP3 antibodies. After first gating the $CD4^+CD25^+$ lymphocytes, the percentage of $FoxP3^+$ cells in this gate was estimated. Tregs were reduced in the LCR rats in both sham and surgery groups compared with HCR rats (Figure 6B).

M2 Macrophage Polarization Was Impaired in the Spleen of LCR Rats

Alternative macrophage activation is a key step for resolution of inflammation and can be detected by the expression of arginase 1 (26). We have shown that IL-10 (Figure 4E) and regulatory T cells (Figure 6B) were reduced in the LCR spleens, both of which may hamper M2 macrophage polarization. Fluorescence-activated cell sorting revealed that $CD11b/c^+$ arginase 1^+ cell populations were reduced in spleens of both sham and surgical (postoperative d 3) LCR rats compared with HCR rats (Figure 6C).

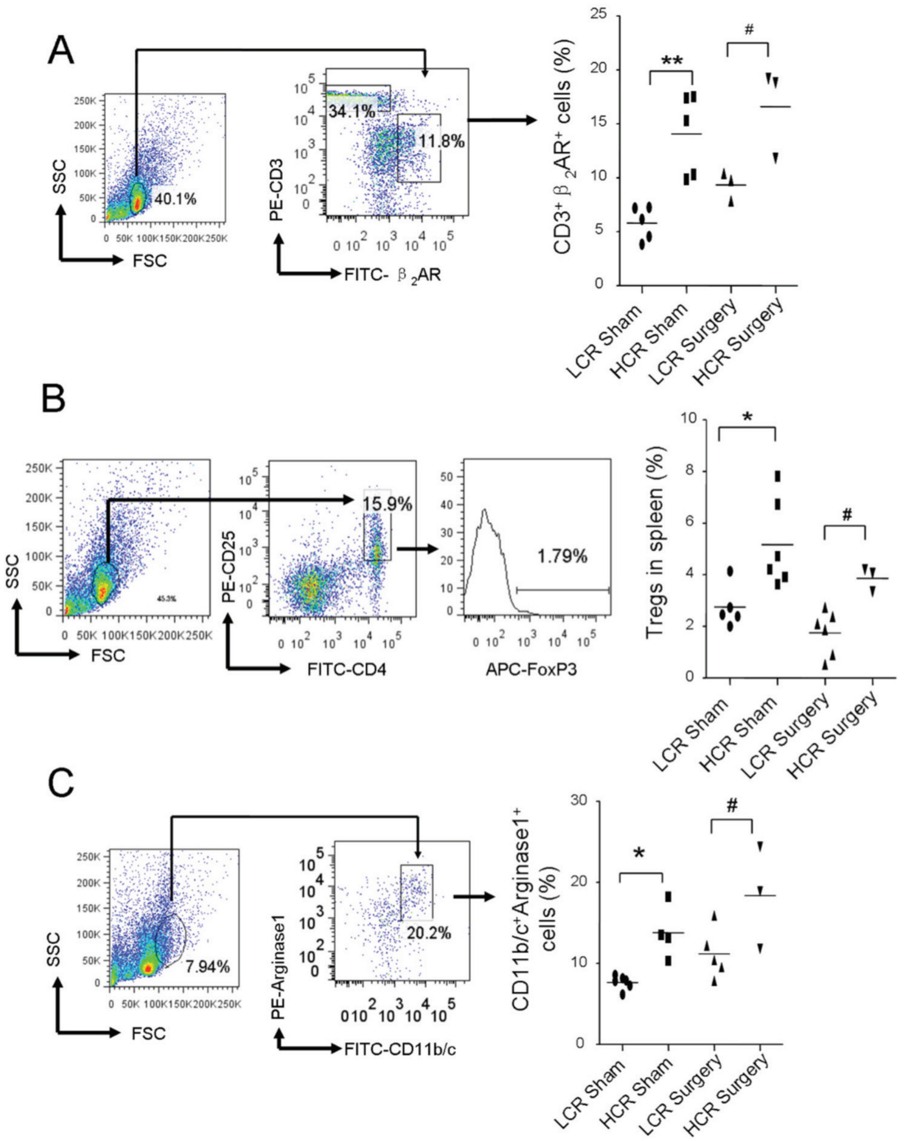

Figure 6. Reduction of β_2 AR–expressing T lymphocytes, regulatory T cells and impairment of M2 macrophage polarization in LCR rats. (A) Analysis of β_2 AR expression in splenic lymphocytes by flow cytometry in LCR and HCR rats under sham and surgical conditions; n = 3–6 in each group; $^{**}p < 0.01$; $^{\#}p < 0.05$. (B) Changes of regulatory T cells in splenic lymphocytes in LCR and HCR rats under sham and surgical conditions; n = 3–6 in each group; $^{*}p < 0.01$; $^{\#}p < 0.05$. (C) Arginase 1 was used as marker to analyze M2 macrophages in splenocytes by flow cytometry in LCR and HCR rats under sham and surgical conditions; n = 3–6 in each group; $^{*}p < 0.01$; $^{\#}p < 0.05$. Each data point represents one independent experiment. *p* values are shown. Values are means ± SD.

Impaired Inflammation Resolution in the Hippocampus in LCR Rats

As indicated in Figure 1, aseptic trauma induces inflammation in the hippocampus through the translocation of bone marrow–derived macrophages that

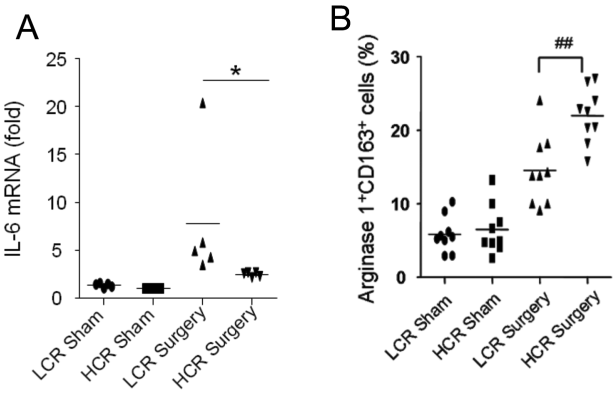

Figure 7. Changes of proinflammatory- and inflammation-resolving mechanisms in the hippocampus of LCR rats after surgery. (A) IL-6 mRNA levels in hippocampus in LCR and HCR rats under sham and surgical conditions at postoperative d 3; $^{*}p < 0.05$ HCR surgery versus LCR surgery. (B) The percentage of M2 macrophages in the hippocampus in LCR and HCR rats under sham and surgical conditions at d 3; $^{\#\#}p < 0.01$ HCR surgery versus LCR surgery; each data point represents one independent experiment. Values are means ± SD.

elaborate and release proinflammatory cytokines that are capable of disrupting long-term potentiation (4–6,30). Neuroinflammation recovers through the action of inflammation-resolving mechanisms including those involving the cholinergic reflex (Figure 2) (4,12,13). We analyzed the changes of proinflammatory (IL-6) levels in the hippocampus as an indicator of neuroinflammation. qPCR analysis revealed significantly more pronounced surgery-induced increase in transcription of the IL-6 mRNA in LCR compared with HCR rats (Figure 7A). Hippocampal staining to detect arginase $1^{+}CD163^{+}$ M2 macrophages revealed a reduction in LCR compared with HCR rats at postoperative d 3 (Figure 7B). These data suggest that there is a defect in alternative macrophage activation that may fail to resolve neuroinflammation in the hippocampus of LCR rats after surgery.

Surgery Led to a Reduction of LXA_4 and an Elevation of LTB_4 Levels in the Circulation of LCR Rats

LXA_4, an eicosanoid product originating from arachidonic acid, potently inhibits NF-κB activity and polarizes macrophages into the alternatively activating proresolving phenotype. Changes in lipoxygenase activity can result in the elaboration of the proinflammatory LTB_4 *in lieu* of LXA_4. In the HCR rats, plasma levels of LXA_4 were 2.2-fold increased and LTB_4 levels were 0.6-fold decreased after surgery. Conversely, in the LCR rats, plasma LXA_4 levels were 0.5-fold reduced and LTB_4 levels were 2.8-fold elevated after surgery (Figures 8A, B). These findings suggest that a humoral inflammation-resolving pathway is impaired in LCR rats.

DISCUSSION

Earlier, we and others have reported on the "physiologic processes" that produce transient postoperative neuroinflammation that results in cognitive decline (4–8,30) (Figure 1). Induction of short-lived neuroinflammation, after release of damage-associated molecular patterns from traumatized tissue, is necessary for the organism's CNS-mediated "sickness behavior," comprising fever, anorexia, somnolence and cognitive impairment. We speculate that this defense mechanism encourages injured animals to remain sedentary, allowing healing rather than risking further injury. Once healing is established, inflammation is dampened and sickness behavior, including cognitive impairment, declines. A series of studies from the Tracey laboratory (12–15) revealed the critical dependence on a cholinergic inflammation-resolving mechanism for attenuating the inflammatory response to infection (Figure 2); recently, we corroborated that this same cholinergic mechanism is necessary when aseptic trauma initiates the inflammatory response (4).

Restoration to normal cognitive function after injury may not occur in pathologic settings, as evidence by the increased likelihood of PCD after surgical procedures in patients with the metabolic syndrome (7,8). Therefore, we decided to explore the underlying abnormalities in the aseptic trauma-induced inflammatory cascade in an animal model of the metabolic syndrome. Because genome-wide association studies have revealed that metabolic syndrome is likely to be a polygenic disorder (31,32), we decided not to use mice with single-gene manipulations; instead, we used rats developed by Koch and Britton in a longstanding National Institutes of Health–sponsored project (18). Starting in 1995, they applied divergent artificial selection for intrinsic low- and high-endurance running capacity starting with a founder population of eight genetically heterogeneous rat strains. A total of 30 generations of selection have produced lines of LCRs and HCRs that differ by sevenfold in treadmill running capacity (18). The LCR rats contain features of the metabolic syndrome, including elevated low-density lipoproteins (LDLs), cholesterol, blood pressure, triglycerides, fasting glucose, insulin, C-reactive protein and visceral adiposity being 100 g heavier than the HCR rats at 12 wks (33). Contrastingly, HCR rats score higher for healthy factors such as maximum volume of O_2 (VO_{2max}) and high-density lipoprotein (34,35).

Earlier, we reported that LCR rats exhibited more severe cognitive decline than HCR rats on postoperative d 7 (20); our current study reveals that this divergence in behavior is already present at postoperative d 3, albeit less pronounced (Figure 3A). Therefore, we have selected this earlier time point at which to interrogate the cholinergic mechanism for inflammation resolution. Surprisingly, we have uncovered statistically significant defects at several steps of this complex pathway. These include (a) decrease of α7 nAChR–expressing CD11b/c$^+$ cells, resulting in hyporesponsiveness to the inhibitory actions of α7 nAChR agonist, thereby increasing NF-κB activity (Figure 4C) and TNF-α production (Figure 4B); (b) decrease of β_2 AR–expressing CD3$^+$ lymphocytes (responsible for the synthesis and release of acetylcholine) (Figure 6A) resulting in the failure of splenic α7 nAChR–expressing CD11b/c$^+$ cells to attenuate NF-κB activation (Figures 4C, D); and (c) reduction of β_2 AR–induced IL-10 release (Figure 4E) and the number of Tregs (Figure 6B) that are capable of impairing M2 macrophage polarization (Figures 6C, 7B).

Because of the possible interaction between dyslipidemia and arachidonic acid metabolism (36), we explored the response to surgery of key eicosanoids that are involved in the regulation of the initiation and resolution of inflammation. On postoperative d 3, LTB_4, a potent proinflammatory eicosanoid, was 2.6-fold higher in the plasma in LCR rats compared with the HCR rats. Conversely, LXA_4, a potent antiinflammatory eicosanoid, was 1.7-fold lower in the plasma in LCR rats than that in the HCR rats. It is possible that these two arachidonic acid products arise from a common precursor that is capable of shunting into either a proinflammatory or proresolving pathway, depending on the regulation of activity of lipoxygenase enzymes in arachidonic acid biotransformation (37,38).

There are two important caveats that need to be considered when interpreting our findings. Although we report numerous mechanistically plausible defects in inflammation resolution that are contemporaneous with exaggerated PCD, we cannot infer that these, individually or collectively, are causally related. Definitive cause-effect relationships must await further studies addressing necessity, sufficiency and reversibility issues; because of the plethora of defects, such mechanistic studies will require a series of experiments involving not only the relatively scarce LCR and HCR rats, but also reagents that either over- or underexpress a particular molecular species. Results from such studies will inform putative therapeutic strategies to rectify defects of the cholinergic and humoral inflammation-resolving pathways.

A second caveat is that we have only investigated a single time point at postoperative d 3; by then, a behavioral phenotype is already present (Figure 3A). Although there is no clear indication when trauma-induced inflammation reverts from initiation to resolution, we had seen evidence at this postoperative time for a behavioral phenotype when inflammation resolution is interrupted by blocking α7 nAChR signaling (4). A high-resolution time-course study will be required to describe the trajectory of changes in the initiation and resolution of trauma-induced inflammation that can result in both early (Figure 3C) as well as the persistent variety of PCD that we earlier reported (20).

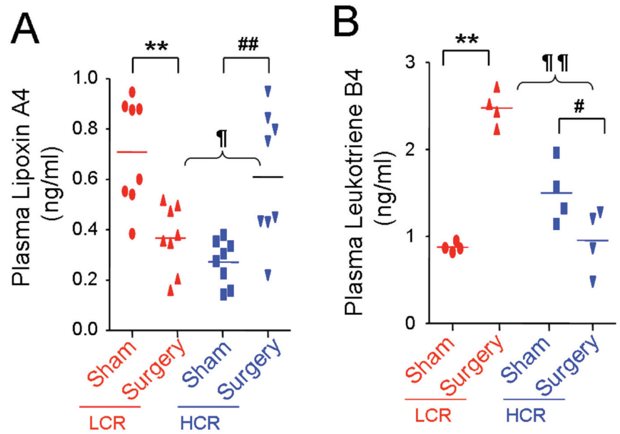

Figure 8. Defects of metabolism of eicosanoids in the LCR rats after surgery caused imbalance of plasma LXA_4 and LTB_4. (A) Changes of plasma LXA_4 among four groups of rats. $^{**}p < 0.01$ for LCR sham versus LCR surgery; $^{\#\#}p < 0.01$ for HCR sham versus HCR surgery; $^{¶}p < 0.05$ for LCR surgery versus HCR surgery; n = 8 in each group. (B) Changes of plasma LTB_4 among four groups of rats. $^{**}p < 0.01$ for LCR sham versus LCR surgery; $^{\#}p < 0.05$ for HCR sham versus HCR surgery; $^{¶¶}p < 0.01$ for LCR surgery versus HCR surgery; n = 4 in each group. Each data point represents one independent experiment. Values are means ± SD.

CONCLUSION

Whether or not these abnormalities in inflammation resolution are responsible for the exaggerated and persistent PCD, we also need to consider whether other inflammation-based postoperative complications, especially chronic postoperative pain (39,40), are also more frequent in surgical patients with metabolic syndrome. Biomarkers are needed to preoperatively identify surgical patients with or without metabolic syndrome that are at risk for nonresolution of inflammation, so that preemptive measures can be attempted to prevent postoperative inflammatory complications.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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