

# UCSF

## UC San Francisco Previously Published Works

### Title

Dysfunction of inflammation-resolving pathways is associated with exaggerated postoperative cognitive decline in a rat model of the metabolic syndrome.

### Permalink

<https://escholarship.org/uc/item/2cb432h6>

### Journal

Molecular Medicine, 18(1)

### Authors

Su, Xiao

Feng, Xiaomei

Terrando, Niccolo

et al.

### Publication Date

2013-02-08

### DOI

10.2119/molmed.2012.00351

### Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

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

Xiao Su,<sup>1,2\*</sup> Xiaomei Feng,<sup>1,3\*</sup> Niccolo Terrando,<sup>1</sup> Yan Yan,<sup>1</sup> Ajay Chawla,<sup>4</sup> Lauren G Koch,<sup>5</sup> Steven L Britton,<sup>5</sup> Michael A Matthay,<sup>4</sup> and Mervyn Maze<sup>1</sup>

<sup>1</sup>Department of Anesthesia and Perioperative Care, University of California, San Francisco, California, United States of America;

<sup>2</sup>Unit of Respiratory Infection and Histopathology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China;

<sup>3</sup>Department of Anesthesiology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; <sup>4</sup>Cardiovascular Research Institute, University of California, San Francisco, California, United States of America; and <sup>5</sup>Department of Anesthesiology, University of Michigan Medical School, Ann Arbor, Michigan, United States of America

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<sub>4</sub> (LXA<sub>4</sub>), 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 LXA<sub>4</sub> and to isolate splenic mononuclear cells (MNCs) to analyze CAP signaling, regulatory T cells (Tregs) and M2 macrophages (M2 M $\phi$ ). Under lipopolysaccharide (LPS) stimulation, tumor necrosis factor (TNF)- $\alpha$  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- $\alpha$  production could not be inhibited by an  $\alpha$ 7 nicotinic acetylcholine receptor agonist, whereas inhibition by the  $\beta$ <sub>2</sub> adrenergic agonist, salmeterol, was significantly less (-35%) than that obtained in HCR rats. Compared to HCR, sham and surgery LCR rats had reduced  $\beta$ <sub>2</sub> adrenergic receptor-expressing T lymphocytes (59%, 44%), Tregs (47%, 54%) and M2 M $\phi$  (45%, 39%); surgical LCR rats' hippocampal M2 M $\phi$  was 66% reduced, and plasma LXA<sub>4</sub> was decreased by 120%. Rats with the metabolic syndrome have ineffective inflammation-resolving mechanisms that represent plausible reasons for the exaggerated and persistent PCD.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2012.00351

## 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 cog-

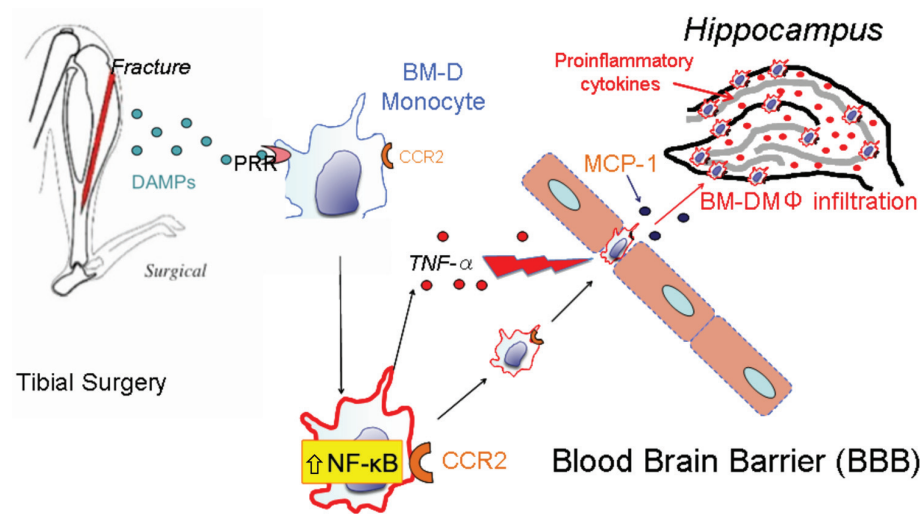
nitive decline before the surgical intervention (3). To address this conundrum, we resorted to rodent models to determine the mechanisms underlying PCD and have defined an inflammatory cascade that is depicted in Figure 1 (4–11).

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 post-ganglionic splenic nerve that terminates in the spleen, where it signals via an  $\alpha$ 7 nicotinic acetylcholine receptor (nAChR) to reduce synthesis of proinflammatory

\*XS and XF contributed equally to this work.

**Address correspondence to** Mervyn Maze or Xiao Su, Department Anesthesia/ Perioperative Care, 521 Parnassus Avenue, Clinic Science, San Francisco, CA 94143. Phone: 415-476-9035; Fax: 415-514-1532. E-mail: mazem@anesthesia.ucsf.edu or xsu@jps.ac.cn.

Submitted December 24, 2012; Accepted for publication January 2, 2013; Epub (www.molmed.org) ahead of print January 3, 2013.



**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- $\kappa$ B-dependent signaling in monocytes, to synthesize and release proinflammatory cytokines, including TNF- $\alpha$ , 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- $\kappa$ B (NF- $\kappa$ B) activity (Figure 2) (12–15). Recently, we showed the importance of this reflex for resolving aseptic trauma-induced neuroinflammation and cognitive decline. Stimulating the  $\alpha 7$  nAChR in macrophages inhibited NF- $\kappa$ B 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  $\beta_2$  adrenergic receptor ( $\beta_2$  AR)-expressing, acetylcholine-synthesizing T lymphocytes in the spleen; and (c) whether eicosanoids involved in inflammation resolution are appropriately bio-transformed. 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  $\alpha 7$  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.

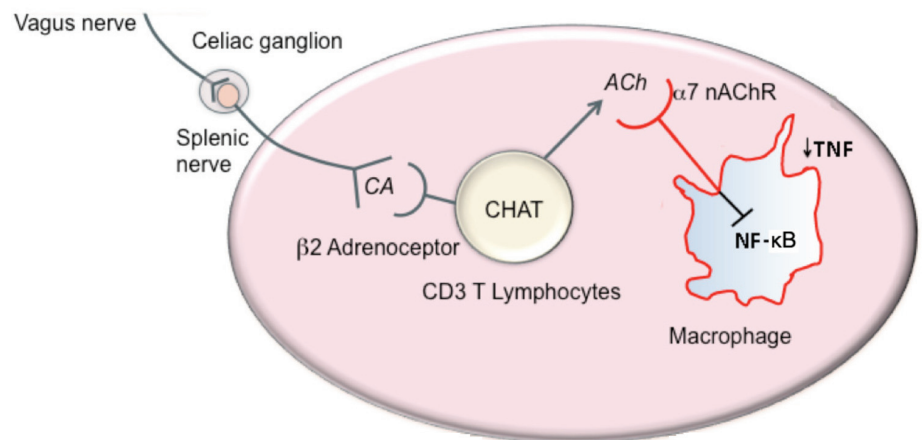
### Aseptic Surgery of Tibia

As previously described (4), animals were anesthetized with 2.1% isoflurane

in 30% FiO<sub>2</sub>. 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<sub>2</sub>) for 20 min in a temperature-controlled anesthetic chamber, shaved and received buprenorphine as described above.

#### Isolation of Mononuclear Cells from Spleen and Peripheral Blood

Spleens were harvested, homogenized and filtered over a 70- $\mu$ m 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<sup>2+</sup> Mg<sup>2+</sup>-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 700g 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 300g and for 10 min at 90g for platelet removal.



**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  $\beta_2$  ARs on CD3 T lymphocytes that contain choline acetyltransferase (CHAT), which is capable of synthesizing the acetylcholine needed to mediate inhibition of macrophage NF- $\kappa$ B activity by signaling through  $\alpha_7$  nAChR.

#### Measurement of NF- $\kappa$ B Activity in the Splenic Mononuclear Cells

Splenic mononuclear cells (MNCs) ( $10^6$ /well) were pretreated with vehicle, PHA 568487 (1  $\mu$ mol/L), MLA (1  $\mu$ mol/L) or PHA 568487 (1  $\mu$ mol/L) + MLA (1  $\mu$ mol/L) and then stimulated with LPS (1  $\mu$ mol/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- $\kappa$ B in the nuclear extract was performed according to the manufacturer's instructions (Active Motif). The levels of p65 NF- $\kappa$ B were expressed by percentage change ([value under LPS stimulation – value under phosphate-buffered saline {PBS} stimulation]/value under PBS stimulation  $\times$  100%).

#### Enzyme-Linked Immunosorbent Assay (ELISA) Measurements for TNF- $\alpha$ and Interleukin-10

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

under LPS stimulation – value under PBS stimulation]/value under PBS stimulation  $\times$  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:  $\beta$ -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  $\Delta$ CT and normalized to *ACTB*. Results are expressed as fold-increases relative to the HCR sham group.

**Enzyme Immunoassay for Plasma Lipoxin A<sub>4</sub> and Leukotriene B<sub>4</sub>**

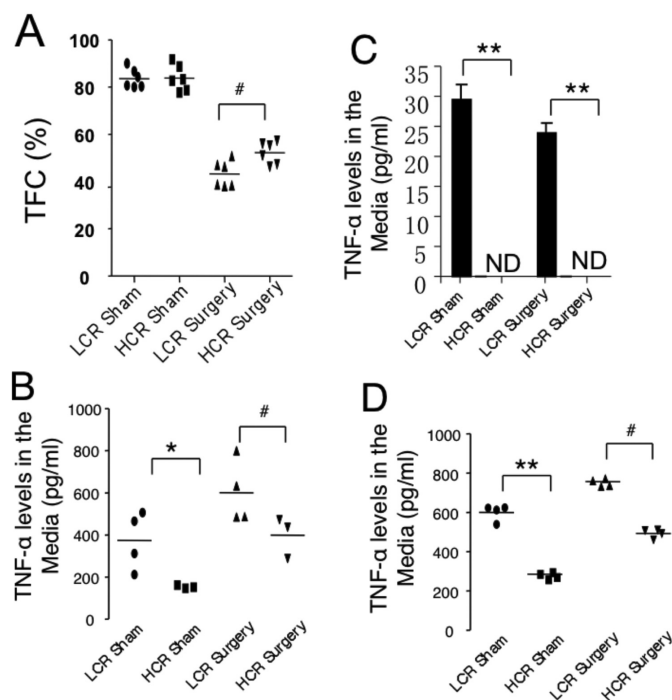
Plasma lipoxin A<sub>4</sub> (LXA<sub>4</sub>) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) were measured by using LXA<sub>4</sub> and LTB<sub>4</sub> enzyme immunoassay following the manufacturer’s instructions (Oxford Biochemical Research, Rochester Hills, MI, USA).

**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  $\beta_2$  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- $\alpha_2$  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  $\alpha 7$  nAChR<sup>+</sup>CD11b<sup>+</sup> cells in the spleens, the splenocytes were labeled with fluorescent-labeled anti- $\alpha 7$  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- $\alpha$  levels were higher in the media of cultured LCR splenic mononuclear cells without LPS challenge; \*\**p* < 0.01 HCR versus LCR. (C) TNF- $\alpha$  levels were reduced in the media of cultured HCR blood mononuclear cells stimulated with LPS (1  $\mu$ mol/L); \**p* < 0.05 HCR sham versus LCR sham; #*p* < 0.05 HCR surgery versus LCR surgery at d 3. (D) TNF- $\alpha$  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  $\pm$  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  $\pm$  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.

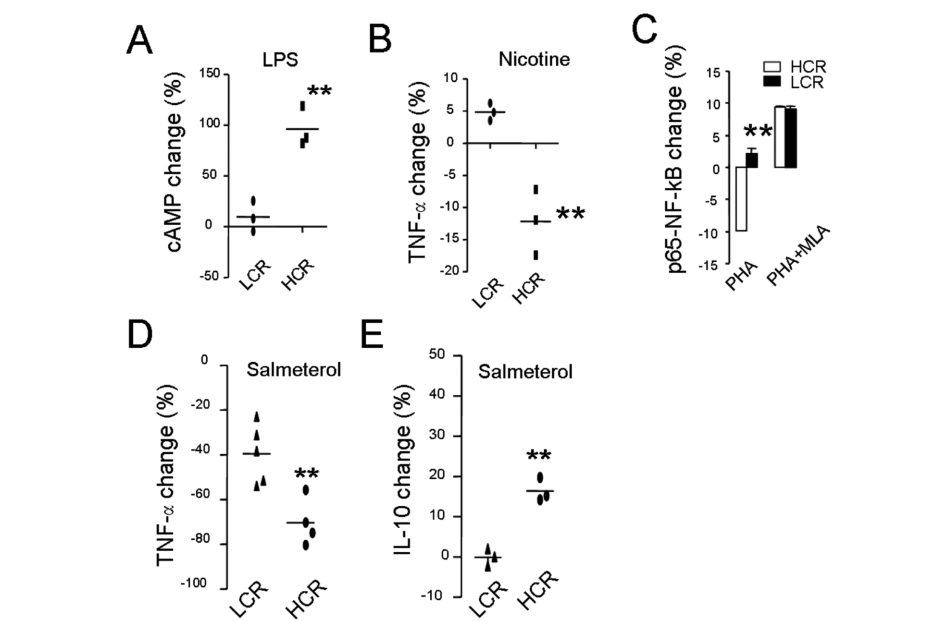
## RESULTS

### 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- $\alpha$  in the medium of cultured splenic MNCs was higher in the LCR cells than that in the HCR cells. Levels of TNF- $\alpha$  did not differ



**Figure 4.** In response to LPS and surgery challenges, impairment of the cholinergic  $\alpha 7$  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  $\mu$ mol/L); \*\**p* < 0.01 HCR compared with LCR. (B) Less inhibitory effects of nicotine on TNF- $\alpha$  levels in LCR splenic MNCs stimulated with LPS; \*\**p* < 0.01 HCR compared with LCR. (C) Less inhibitory effects of PHA 568487 (PHA, 1  $\mu$ mol/L) on p65 NF- $\kappa$ B 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  $\mu$ mol/L) counteracted the inhibitory effects of PHA 568487. (D) Less inhibitory effects of salmeterol on TNF- $\alpha$  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  $\mu$ mol/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  $\pm$  SD.

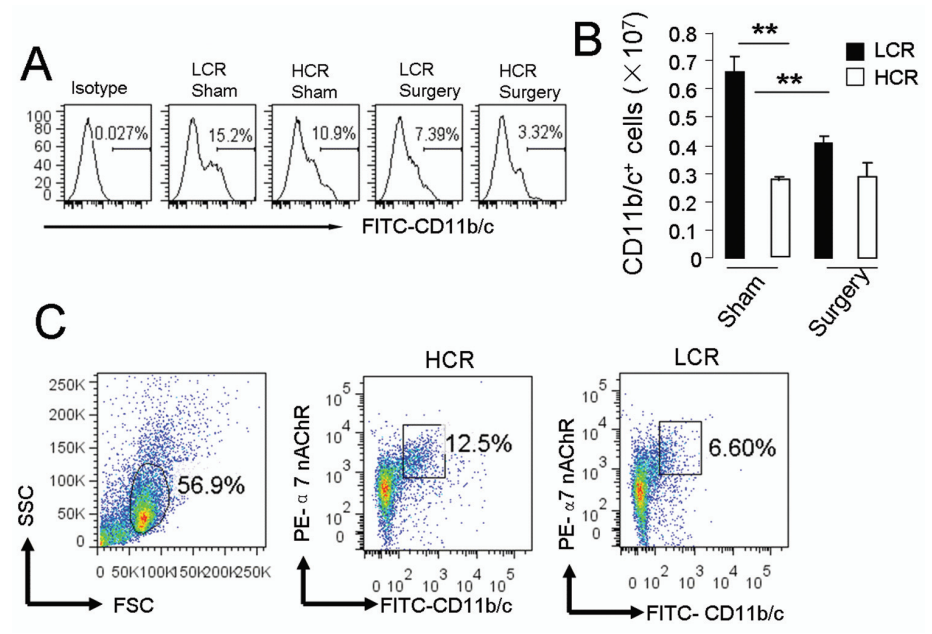
between LCR sham and surgery (Figure 3B); this is unsurprising, since the postoperative rise and fall in TNF- $\alpha$  is completed by 24 h (6,22). After stimulation with LPS, both LCR peripheral blood and spleen MNCs produced significantly more TNF- $\alpha$  compared with HCR rat spleen MNCs in both sham and surgery groups (Figures 3C, D), indicating that surgery in LCR rats primes proinflammatory signaling and that TNF- $\alpha$  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 (Fig-

ure 3A) occurs at a time that bone marrow-derived macrophages were more proinflammatory (Figures 3B–D).

### Dysfunction of Cholinergic Antiinflammatory Pathway in the LCR Rats

cAMP, a second messenger required for acetylcholine- $\alpha 7$  nAChR signaling in the splenic MNCs (23,24), can be stimulated by LPS (1  $\mu$ mol/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  $\alpha 7$  nAChR signaling.

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



**Figure 5.** M1 macrophages were elevated and proresolving  $\alpha 7$  nAChR<sup>+</sup>CD11b/c<sup>+</sup> 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<sup>+</sup> 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  $\alpha 7$  nAChR<sup>+</sup>CD11b/c<sup>+</sup> cells in the splenocytes. Values are means  $\pm$  SD.

inflammatory reflex (12–15). Therefore, we tested whether  $\alpha 7$  nAChR signaling is capable of inhibiting LPS-induced TNF- $\alpha$  production in the splenic MNCs. Nicotine, 1  $\mu$ mol/L, a nonselective  $\alpha 7$  nAChR agonist, inhibited TNF- $\alpha$  in the HCR splenic MNCs stimulated with LPS, but failed to affect TNF- $\alpha$  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  $\mu$ mol/L), a selective  $\alpha 7$  nAChR agonist (4,25) to test whether activation of  $\alpha 7$  nAChR could reduce NF- $\kappa$ B activation when challenged with LPS for 1 h. PHA 568487 blocked NF- $\kappa$ B p65 subunit nuclear translocation in the HCR splenic MNCs stimulated with LPS; conversely, NF- $\kappa$ B p65 subunit nuclear translocation was not reduced in the LCR splenic MNCs, suggesting that there is dysfunction in the  $\alpha 7$  nAChR signaling pathway. To establish the specificity of this response to the  $\alpha 7$  nAChR, we confirmed

that the  $\alpha 7$  nAChR antagonist, MLA, blocked the PHA 568487 effect on p65 NF- $\kappa$ B nuclear translocation (Figure 4C).

Agonists of  $\beta 2$  AR promote splenic choline acetyltransferase-expressing lymphocytes to synthesize acetylcholine (13), which is needed to inhibit NF- $\kappa$ B activity and the downstream synthesis and release of proinflammatory cytokines, including TNF- $\alpha$ . To assess this pathway, isolated splenocytes were pretreated with the selective  $\beta 2$  AR agonist salmeterol (1  $\mu$ mol/L) and then stimulated with LPS for 4 h to release TNF- $\alpha$  into the medium; salmeterol more effectively inhibited TNF- $\alpha$  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  $\beta 2$  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<sup>+</sup> 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<sup>+</sup> cells comprised a larger percentage of splenocytes from LCR rats compared with HCR rats (Figure 5A). By multiplying the percentage of CD11b/c<sup>+</sup> 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 decreased 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  $\alpha 7$  nAChR-expressing M1 macrophages (CD11b/c<sup>+</sup>) that are responsive to the acetylcholine released by the vagal reflex, we considered whether reduced expression of  $\alpha 7$  nAChR in the M1 macrophages may be responsible for the attenuation of the antiinflammatory effects of the cholinergic  $\alpha 7$  nAChR pathway in LCR rats (Figures 5B, C). To test this, we labeled splenocytes isolated from LCR and HCR rats with both anti- $\alpha 7$  nAChR and CD11b/c antibodies. The whole cell population was gated during flow cytometry analysis. The  $\alpha 7$  nAChR<sup>+</sup>CD11b/c<sup>+</sup> 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.



### $\beta_2$ AR-Expressing CD3 Lymphocytes Are Reduced in the LCR Spleen

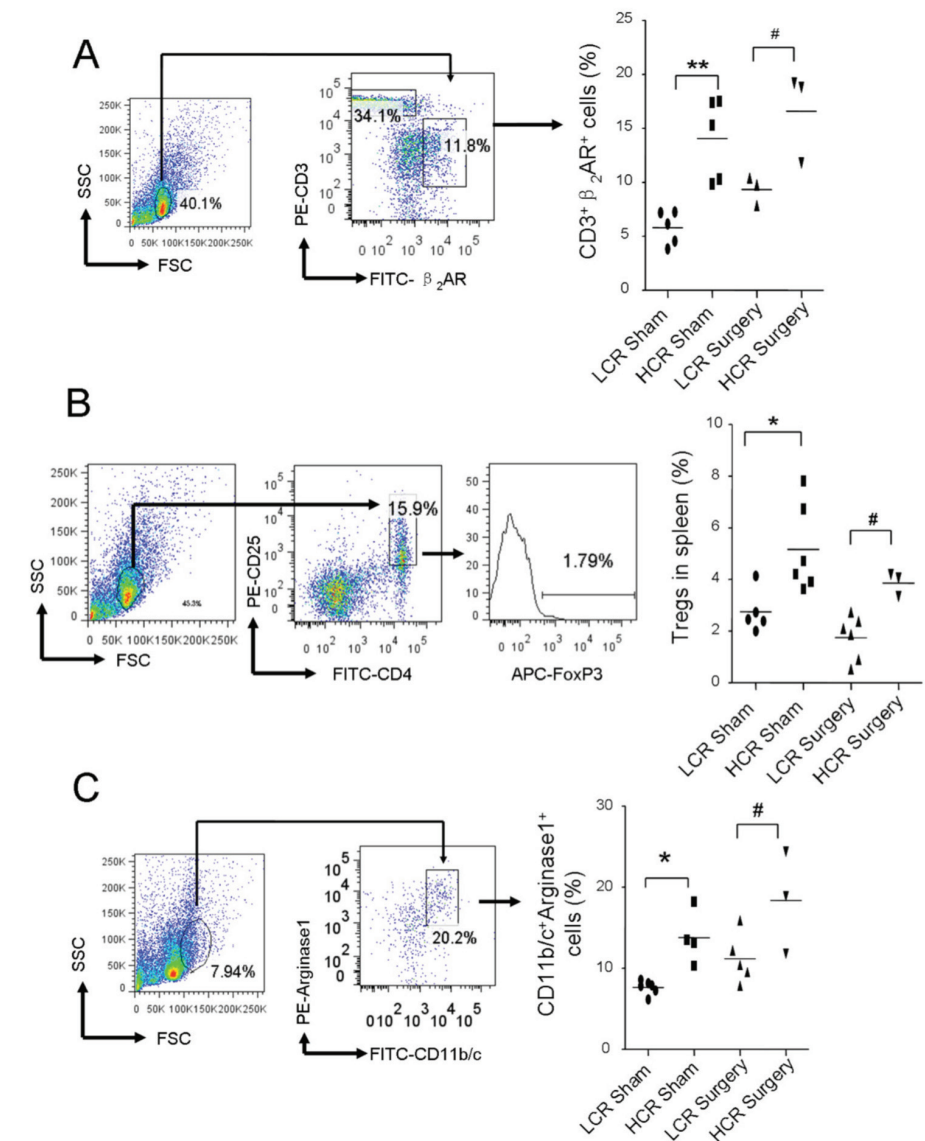
$\beta_2$  AR-expressing CD3<sup>+</sup> lymphocytes are acetylcholine-synthesizing cells in the spleen that are required for relaying the vagally mediated antiinflammatory signal (13). Because salmeterol had less inhibitory effect on TNF- $\alpha$  (Figure 4D), we explored whether there is a reduction in the population of  $\beta_2$  AR-expressing lymphocytes (CD3<sup>+</sup>) in LCR rat splenocytes by using anti-rat CD3 and  $\beta_2$  AR antibodies and flow cytometry.  $\beta_2$  AR<sup>+</sup>CD3<sup>+</sup> lymphocytes were reduced in both LCR sham and surgery groups (Figure 6A), which suggest that the reduced  $\beta_2$  AR<sup>+</sup>CD3<sup>+</sup> lymphocyte population may contribute to less attenuation of inflammation through the  $\alpha 7$  nAChR pathway (Figures 4D, E).

### Regulatory T Cells (CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) 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  $\alpha 7$  nAChR plays a critical role in regulating immunosuppressive function of CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> lymphocytes, the percentage of FoxP3<sup>+</sup> 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



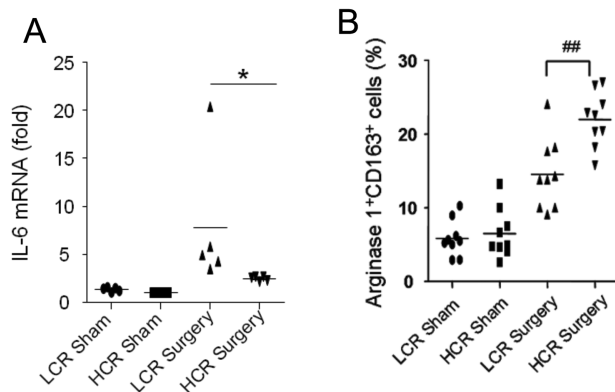
**Figure 6.** Reduction of  $\beta_2$  AR-expressing T lymphocytes, regulatory T cells and impairment of M2 macrophage polarization in LCR rats. (A) Analysis of  $\beta_2$  AR expression in splenic lymphocytes by flow cytometry in LCR and HCR rats under sham and surgical conditions;  $n = 3-6$  in each 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  $\pm$  SD.

macrophage polarization. Fluorescence-activated cell sorting revealed that CD11b/c<sup>+</sup> arginase 1<sup>+</sup> cell populations were reduced in spleens of both sham and surgical (postoperative d 3) LCR rats compared with HCR rats (Figure 6C).

### 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  $\pm$  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<sup>+</sup>CD163<sup>+</sup> 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<sub>4</sub> and an Elevation of LTB<sub>4</sub> Levels in the Circulation of LCR Rats

LXA<sub>4</sub>, an eicosanoid product originating from arachidonic acid, potentially inhibits NF- $\kappa$ B activity and polarizes macrophages into the alternatively activating proresolving phenotype. Changes

in lipoxygenase activity can result in the elaboration of the proinflammatory LTB<sub>4</sub> in lieu of LXA<sub>4</sub>. In the HCR rats, plasma levels of LXA<sub>4</sub> were 2.2-fold increased and LTB<sub>4</sub> levels were 0.6-fold decreased after surgery. Conversely, in the LCR rats, plasma LXA<sub>4</sub> levels were 0.5-fold reduced and LTB<sub>4</sub> 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 se-

ries 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<sub>2</sub> (VO<sub>2max</sub>) 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

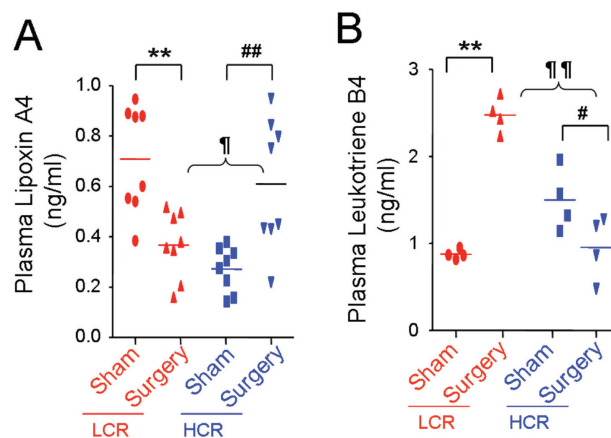
have selected this earlier time point at which to interrogate the cholinergic mechanism for inflammation resolution. Surprisingly, we have uncovered statistically significant defects at several steps of this complex pathway. These include (a) decrease of  $\alpha 7$  nAChR-expressing CD11b/c<sup>+</sup> cells, resulting in hypo-responsiveness to the inhibitory actions of  $\alpha 7$  nAChR agonist, thereby increasing NF- $\kappa$ B activity (Figure 4C) and TNF- $\alpha$  production (Figure 4B); (b) decrease of  $\beta_2$  AR-expressing CD3<sup>+</sup> lymphocytes (responsible for the synthesis and release of acetylcholine) (Figure 6A) resulting in the failure of splenic  $\alpha 7$  nAChR-expressing CD11b/c<sup>+</sup> cells to attenuate NF- $\kappa$ B activation (Figures 4C, D); and (c) reduction of  $\beta_2$  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<sub>4</sub>, a potent proinflammatory eicosanoid, was 2.6-fold higher in the plasma in LCR rats compared with the HCR rats. Conversely, LXA<sub>4</sub>, 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  $\alpha 7$  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<sub>4</sub> and LTB<sub>4</sub>. (A) Changes of plasma LXA<sub>4</sub> 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<sub>4</sub> 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.

## ACKNOWLEDGMENTS

The LCR-HCR rat model system was funded by the National Center for Research Resources grant R24 RR017718 (to LG Koch and SL Britton) and is currently supported by the Office of Research Infrastructure Programs/OD grant ROD012098A (to LG Koch and SL Britton) from the National Institutes of Health. SL Britton was also supported by National Institutes of Health grant RO1 DK077200. We acknowledge the expert care of the rat colony provided by Molly Kalahar and

Lori Gilligan. The LCR and HCR model can be made available for collaborative study (contact brittons@umich.edu or lgkoch@umich.edu). This work was also supported by Parker B Francis Fellowship (X Su), the Knowledge Innovation Program of the CAS (Y114P11209, X Su), the National Natural Science Foundation of China (81270139, X Su), and funds from the Department of Anesthesia, University of California San Francisco.

## 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.

## REFERENCES

- Steinmetz J, et al. (2009) Long-term consequences of postoperative cognitive dysfunction. *Anesthesiology*. 110:548–55.
- Koster S, Hensens AG, van der Palen J. (2009) The long-term cognitive and functional outcomes of postoperative delirium after cardiac surgery. *Ann. Thorac. Surg.* 87:1469–74.
- Crosby G, Culley DJ, Hyman BT. (2011) Preoperative cognitive assessment of the elderly surgical patient: a call for action. *Anesthesiology*. 114:1265–8.
- Terrando N, et al. (2011) Resolving postoperative neuroinflammation and cognitive decline. *Ann. Neurol.* 70:986–95.
- Degos V, et al. (2013) Hippocampal recruitment of systemic macrophages plays a causal role in postoperative memory dysfunction in mice. *Anesthesiology*. In Press.
- Terrando N, et al. (2010) Tumor necrosis factor- $\alpha$  triggers a cytokine cascade yielding postoperative cognitive decline. *Proc. Natl. Acad. Sci. U. S. A.* 107:20518–22.
- Cibelli M, et al. (2010) Role of interleukin-1 $\beta$  in postoperative cognitive dysfunction. *Ann. Neurol.* 68:360–8.
- Terrando N, et al. (2011) Perioperative cognitive decline in the aging population: Mayo Clinic proceedings. *Mayo Clinic*. 86:885–93.
- Fidalgo AR, et al. (2011) Systemic inflammation enhances surgery-induced cognitive dysfunction in mice. *Neurosci. Lett.* 498:63–6.
- Wan, et al. (2007) Postoperative impairment of cognitive function in rats: a possible role for cytokine-mediated inflammation in the hippocampus. *Anesthesiology*. 106:436–43.
- Vizcaychipi MP, et al. (2011) Heat shock protein overexpression prevents early postoperative memory decline after orthopedic surgery during general anesthesia in mice. *Anesthesiology*. 114:891–900.
- Tracey KJ. (2009) Reflex control of immunity. *Nat. Rev. Immunol.* 9:418–28.
- Rosas-Ballina M, et al. (2011) Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science*. 334:98–101.
- Pavlov VA, Tracey KJ. (2012) The vagus nerve and the inflammatory reflex-linking immunity and metabolism. *Nat. Rev. Endocrinol.* 8:743–54.
- Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ. (2012) Rethinking inflammation: neural circuits in the regulation of immunity. *Immunol. Rev.* 248:188–204.
- Hudetz JA, Patterson KM, Amole O, Riley AV, Pagel PS. (2011) Postoperative cognitive dysfunction after noncardiac surgery: effects of metabolic syndrome. *J. Anesthesia*. 25:337–44.
- Hudetz JA, Patterson KM, Iqbal Z, Gandhi SD, Pagel PS. (2011) Metabolic syndrome exacerbates short-term postoperative cognitive dysfunction in patients undergoing cardiac surgery: results of a pilot study. *J. Cardiothorac. Vasc. Anesth.* 25:282–7.
- Koch LG, Britton SL. (2001) Artificial selection for intrinsic aerobic endurance running capacity in rats. *Physiol. Genomics*. 5:45–52.
- Wisloff U, et al. (2005) Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. *Science*. 307:418–20.
- Feng X, et al. (2013) Surgery results in exaggerated and persistent cognitive decline in a rat model of the Metabolic Syndrome. *Anesthesiology*. In Press.
- Koch LG, Britton SL, Wisloff U. (2012) A rat model system to study complex disease risks, fitness, aging, and longevity. *Trends Cardiovasc. Med.* 22:29–34.
- Risnes I, et al. (2003) Changes in the cytokine network and complement parameters during open heart surgery. *Interact. Cardiovasc. Thorac. Surg.* 2:19–24.
- Higgins LS, Berg DK. (1988) Cyclic AMP-dependent mechanism regulates acetylcholine receptor function on bovine adrenal chromaffin cells and discriminates between new and old receptors. *J. Cell. Biol.* 107:1157–65.
- Oshikawa J, et al. (2003) Nicotinic acetylcholine receptor alpha 7 regulates cAMP signal within lipid rafts. *Am. J. Physiol. Cell Physiol.* 285:C567–74.
- Su X, Matthay MA, Malik AB. (2010) Requisite role of the cholinergic alpha7 nicotinic acetylcholine receptor pathway in suppressing Gram-negative sepsis-induced acute lung inflammatory injury. *J. Immunol.* 184:401–10.
- Lawrence T, Natoli G. (2011) Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat. Rev. Immunol.* 11:750–61.
- O'Mahony C, van der Kleij H, Bienenstock J, Shanahan F, O'Mahony L. (2009) Loss of vagal anti-inflammatory effect: in vivo visualization and adoptive transfer. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 297:R1118–26.
- Wang DW, et al. (2010) Stimulation of alpha7 nicotinic acetylcholine receptor by nicotine increases suppressive capacity of naturally occurring CD4+CD25+ regulatory T cells in mice in vitro. *J. Pharmacol. Exp. Ther.* 335:553–61.
- Tiemessen MM, et al. (2007) CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc. Natl. Acad. Sci. U. S. A.* 104:19446–51.
- Barrientos, et al. (2012) Intracisternal interleukin-1 receptor antagonist prevents postoperative cognitive decline and neuroinflammatory response in aged rats. *J. Neurosci.* 32:14641–8.
- Edwards KL, Hutter CM, Wan JY, Kim H, Monks SA. (2008) Genome-wide linkage scan for the metabolic syndrome: the GENNID study. *Obesity*. 16:1596–601.
- Joy T, Lahiry P, Pollex RL, Hegele RA. (2008) Genetics of metabolic syndrome. *Curr. Diab. Rep.* 8:141–8.
- Noland RC, et al. (2007) Artificial selection for high-capacity endurance running is protective against high-fat diet-induced insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* 293:E31–41.
- Gonzalez NC, et al. (2006) Continued divergence in VO<sub>2</sub>max of rats artificially selected for running endurance is mediated by greater convective blood O<sub>2</sub> delivery. *J. Appl. Physiol.* 101:1288–96.
- Kivela R, et al. (2010) Gene expression centroids that link with low intrinsic aerobic exercise capacity and complex disease risk. *FASEB J.* 24:4565–74.
- Schrör K. (1990) Platelet reactivity and arachidonic acid metabolism in type II hyperlipoproteinemia and its modification by cholesterol-lowering agents. *Eicosanoids*. 3:67–73.
- Garrick R, Goodman A, Shen ST, Ogunc S, Wong PY. (1991) Regulation of lipoxins (LX) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) production in rat mesangial cells (MC). *Adv. Prostaglandin Thromboxane Leukot. Res.* 21B:701–6.
- Rao NL, et al. (2007) Anti-inflammatory activity of a potent, selective leukotriene A<sub>4</sub> hydrolase inhibitor in comparison with the 5-lipoxygenase inhibitor zileuton. *J. Pharmacol. Exp. Ther.* 321:1154–60.
- Mutso AA, et al. (2012) Abnormalities in hippocampal functioning with persistent pain. *J. Neurosci.* 32:5747–56.
- Duric V, McCarson KE. (2007) Neurokinin-1 (NK-1) receptor and brain-derived neurotrophic factor (BDNF) gene expression is differentially modulated in the rat spinal dorsal horn and hippocampus during inflammatory pain. *Mol. Pain*. 3:32.