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Vagus nerve regulates the phagocytic and secretory activity of resident macrophages in the liver

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ABSTRACT

The gastrointestinal (GI) tract harbors commensal microorganisms as well as invasive bacteria, toxins and other pathogens and, therefore, plays a pivotal barrier and immunological role against pathogenic agents. The vagus nerve is an important regulator of the GI tract-associated immune system, having profound effects on inflammatory responses. Among GI tract organs, the liver is a key site of immune surveillance, as it has a large population of resident macrophages and receives the blood drained from the guts through the hepatic portal circulation. Although it is widely accepted that the hepatic tissue is a major target for vagus nerve fibers, the role of this neural circuit in liver immune functions is still poorly understood. Herein we used *in vivo* imaging techniques, including confocal microscopy and scintigraphy, to show that vagus nerve stimulation increases the phagocytosis activity by resident macrophages in the liver, even on the absence of an immune challenge. The activation of this neural circuit in a non-lethal model of sepsis optimized the removal of bacteria in the liver and resulted in the production of anti-inflammatory and pro-regenerative cytokines. Our findings provide new insights into the neural regulation of the immune system in the liver.

1. Introduction

The gastrointestinal tract integrates its classical functions in an environment harboring a large population of commensal microorganisms, invasive pathogens and toxins, all of which can impact the immune system (Vanner et al., 2016). Paralleling the physiological regulation of secretion, absorption and motility, the gastrointestinal system has to control the immune system to avoid both translocation of microorganisms/toxins and triggering of inflammatory responses (Jenne and Kubes, 2013). This balance is achieved through the action of neural circuits, which are involved in sensing harmful stimuli and in the onset and integration of appropriate immune responses (Chavan et al., 2017; Tracey, 2016).

Among these neural circuits, the one mediated by the vagus nerve is

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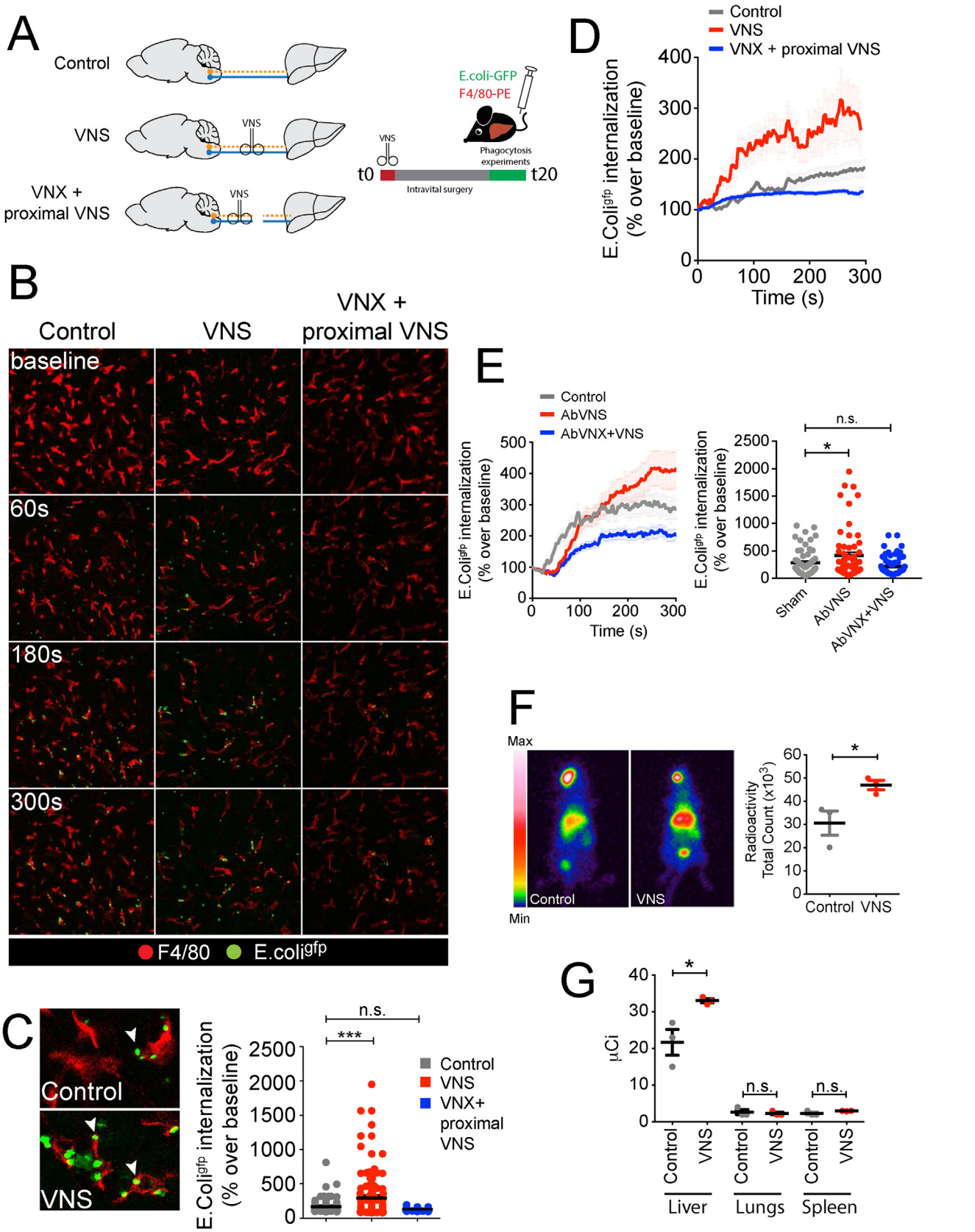

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Fig. 1. Vagus nerve stimulation (VNS) increases phagocytosis activity by Kupffer cells. (A) Experimental design and timeline. t0 = begin of experiment; t20 = total duration of the experiment in minutes (B) Representative *in vivo* confocal images of the phagocytosis assays. (C) Quantification of *E. coli*^{gfp} in Kupffer cells 300 s after loading (n = 50 cells/group; 3 mice/group; ***$P < 0.001$; n.s. = not significant; One-way ANOVA with Newman-Keuls Multiple Comparison Test; error bars = mean ± s.e.m). (D) Time course of the dynamics of phagocytosis with cervical vagus nerve stimulation (VNS) or vagotomy (VNX) with proximal stimulation. Data is presented as mean ± s.e.m. (E) Time course of the dynamics of phagocytosis and quantification of the data with abdominal vagus nerve stimulation (AbVNS) or abdominal vagotomy followed by nerve stimulation (AbVNX + VNS). Data is presented as mean ± s.e.m (n = 20 cells/group; 3 mice/group; *$P < 0.05$; n.s. = not significant; One-way ANOVA with Newman-Keuls Multiple Comparison Test; error bars = mean ± s.e.m.) (F) Scintillation imaging of mice injected with ^{99m}Tc-labelled colloidal tin (n = 3/group; *$P < 0.05$; unpaired Student's *t*-test; error bars = mean ± s.e.m). (G) *Ex vivo* determination of radioactive signals in the liver, lungs and spleen of mice injected with ^{99m}Tc-labelled colloidal tin (n = 3/group; *$P < 0.05$; unpaired Student's *t*-test; n.s. = not significant; error bars = mean ± s.e.m; μCi = micro Curie).

of special interest for the gastrointestinal tract. First, because the vagus nerve is a major sensory and efferent nerve to the gastrointestinal organs (Berthoud and Neuhuber, 2000; Berthoud et al., 1991). Second, because stimulation of the efferent arm of the vagal circuitry counterbalances tissue-damaging inflammation through the control of pro-inflammatory cytokines release and promotion of immune cells activation and differentiation towards a pro-regenerative phenotype (Borovikova et al., 2000; Bonaz et al., 2018; Chavan et al., 2017; Gabanyi et al., 2016; Koopman et al., 2016; Pavlov and Tracey, 2017). The classical cholinergic anti-inflammatory reflex involves the bidirectional communication between peripheral physiological systems and the central nervous system (CNS), in which the stimuli are sensed by afferent vagal fibers that transmit this information to the nucleus of the solitary tract within the CNS (Goehler et al., 2000). After processing in the CNS, the responses to these stimuli are effected by efferent vagal fibers originated from the dorsal motor nucleus of the vagus nerve or the nucleus ambiguous (Goehler et al., 2000; Pavlov and Tracey, 2017; Tracey, 2016).

Interestingly, a great number of the subdiaphragmatic vagus nerve fibers form the hepatic branch of the nerve and innervates the liver (Berthoud, 2004; Berthoud et al., 1991; McCuskey, 2004). Therefore, among the organs within the abdominal cavity, the liver is a major target of vagus nerve. In addition, the hepatic environment harbors one of the largest populations of resident macrophages (Kupffer cells) within the organism (Jenne and Kubes, 2013). These cells are involved in the elimination of invasive pathogens by phagocytosis (Broadley et al., 2016; David et al., 2016) and also in the secretion of inflammatory mediators that drive local immune responses. In this work, we demonstrate, *in vivo*, a novel branch of the vagus nerve anti-inflammatory circuit that increases the phagocytic activity by Kupffer cells, in addition to the classical action on the modulation of cytokine production. This neural reflex also optimizes liver bacterial clearance during sepsis and, therefore, constitutes an important pathway to regulate body's immune surveillance.

2. Methods

2.1. Ethical approval

All experimental procedures were performed in accordance with protocols approved by the institutional Animal Use Committee (CEUA/UFMG, protocol 311/2016) and the Brazilian legislation of animal care and experimentation. None of the viable animals were excluded from the analyses.

2.2. Animals and surgical procedures

Female C57/Bl6J mice (8–10 weeks-old) used in this study were obtained from the animal facility of the Federal University of Minas Gerais (*Centro de Bioterismo, CEBIO/UFMG*). Dr. Marco Antônio Prado, from the Robarts Research Institute (University of Western Ontario, Canada) kindly provided the VAChT KD^{hom} and WT mice used in the experiments (background C57/Bl6J-A – agouti). The ChAT-TdTomato animals used in this study were supplied by Dr. Ivan de Araujo e Dr. Wenfei Han from the Icahn School of Medicine at Mount Sinai (NY). Animals were housed under a 12-hour light/dark cycle at 25 °C, with unrestricted access to food and water, at the animal housing facility of the Institute for Biological Sciences/UFMG.

Mice were anesthetized with ketamine and xylazine (80 mg/Kg and 15 mg/Kg, respectively; i.p.), submitted to a ventral medial laparotomy through the *linea alba* and to one of the surgical procedures as follows. (1) Removal of the celiac ganglion (CGX), was made after the identification of the ganglion as an irregular shaped mass of nerve tissue located between the inferior phrenic artery and the medial adrenal artery with its subsequent excision; (2) splenectomy (SPX) was performed after ligation of spleen blood (Huston et al., 2006) or (3) bilateral adrenalectomy (ADX) (Torres-Rosas et al., 2014) in which adrenal glands were removed with a forceps. Adrenalectomized mice were allowed free access to 0.9% NaCl solution to avoid electrolyte loss. (4) Vagotomy (VNX) was performed after a midline incision cervical region for the isolation of the left cervical trunk of the vagus nerve (Borovikova et al., 2000). Then, the nerve was ligated with a 4-0 silk sutures and transected. Control groups were submitted to the same surgical procedures without the removal of any organ. All animals were allowed recovering for 7 days before the experiments.

For the electrical vagus nerve stimulation (VNS) in mice, a small ventral incision was made at the cervical region to expose the trachea and the left carotid artery, which is adjacent to the left cervical trunk of the vagus nerve. After isolation from the surrounding tissues, the nerve was placed across a platinum electrode connected to a stimulation module and constant voltage stimuli (1 V, 0.1 ms, 5 Hz) (Bassi et al., 2017) were applied to the nerve for 2 min before the experiments. In the VNX + VNS group, this procedure was performed in the segment of the vagus nerve above the section (proximal VNS) (Fig. 1A).

We further performed the phagocytosis assays after abdominal vagus nerve stimulation (AbVNS) or abdominal vagotomy (AbVNX) followed by VNS. For these experiments, we identified the ventral (or anterior) trunk of the nerve, which is contiguous with the left cervical vagus, branches into the common hepatic nerve and is, therefore, the main source of vagal fibers to the liver through the hepatic branch proper (Berthoud, 2004; Berthoud and Neuhuber, 2000; Simons et al., 1998). In the case of the AbVNX + VNS, the electrical stimulation was performed on the left cervical branch of the nerve.

2.3. Pharmacological inhibition of acetylcholine receptors

Acetylcholine nicotinic and muscarinic receptors antagonists, mecamylamine (Meca) and methylatropine (MetA), respectively, were dissolved in sterile saline and administered (1 mg/Kg) (Mao et al., 2015; Zachariou et al., 2001) through a single intraperitoneal injection 10 min before VNS.

2.4. Intravital microscopy and phagocytosis analysis

The impact of VNS on the phagocytosis activity by Kupffer cells was analyzed by intravital microscopy as previously described (Marques et al., 2015). Briefly, anesthetized mice received an intravenous single dose of anti-F4/80 conjugated with phycoerythrin (4 mg, clone BM8, eBiosciences, USA) 10 min before the surgery. Then, animals were submitted to a midline laparotomy, the abdominal muscles were

removed using a cautery and the animals were placed in the right dorsolateral position to exteriorize the right lobe of the liver. Mice were finally placed on the confocal stage, injected with 5×10^7 *Escherichia coli* GFP (i.v.; *E. coli*^{gfp}; ATCC 25922GFP) and imaged under confocal microscopy (Nikon A1R, Nikon Instruments, USA) for 5 min and 40× Nikon Plan Apo objective (David et al., 2016).

Cell number and bacterial catching by Kupffer cells quantifications were made using the image-processing package Fiji (Schindelin et al., 2012).

2.5. Experiments with ^{99m}Tc-labeled colloidal tin and scintillation imaging

For the experiments involving the usage of radiopharmaceuticals, we first eluted a sterile solution of sodium pertechnetate ($Na^{99m}TcO_4$, 99mTc) with activity of 148 MBq (4 mCi) from the molybdenum-technetium generator (^{99}Mo/^{99m}Tc; IPEN/CNEN/UFMG). The, colloidal tin was incubated and labeled with ^{99m}Tc for 15 min at room temperature. We used radiopharmaceuticals with radiochemical purity levels of over 90% in all experiments.

For scintillation imaging, anesthetized mice were injected intravenously with 0.1 ml (3 MBq) of ^{99m}Tc-tin. Following injection, the animals were placed in a gamma camera (Mediso, Hungary) and imaged using a low energy high-resolution collimator for 10 min. The liver was traced in each image for total radioactivity count. Then, mice were euthanized and the liver, spleen and lungs dissected for *ex vivo* determination of technetium radioactivity in a radioisotope dose meter (Capintec, USA).

2.6. Isolation of Kupffer cells

Mice were anesthetized and submitted to the VNS protocol previously described. Then, the liver was removed, minced in small fragments and digested in RPMI medium supplemented with 2% fetal bovine serum and collagenase VIII (Sigma-C2139, at a concentration of 1 mg/ml). Samples were incubated under agitation for 1 h at 37 °C. Next, a solution of BSA 0.5%, EDTA 2 mM in PBS was added to the samples. After digestion, liver homogenates were submitted to differential centrifugation steps to remove hepatocytes and to obtain liver non-parenchymal cells (LNPCs).

Kupffer cells were isolated from the LNPCs fraction. For this, we incubated LNPCs with anti-F4/80 conjugated with phycoerythrin (clone BM8, eBiosciences, USA) for 30 min at 4 °C. Then, samples were incubated with an antibody anti-phycoerythrin conjugated with magnetic beads (clone E31-1459, BD Biosciences, USA) and placed onto a strong magnet (iMag, BD Biosciences, USA) for 15 min. The purity and efficiency of the extraction was addressed by flow cytometry (Accuri™ C6 cytometer, BD biosciences, USA). FlowJo (FlowJo, USA) was used to analyze the results. Isolated Kupffer Cells were frozen in liquid nitrogen and stored at −80 °C for qRT-PCR.

2.7. Neuro-immune interaction studies

The cervical left trunk of the vagus nerve was exposed as previously described and placed over a Parafilm piece. Then, a cotton bud was saturated with DiO 421 neurotracer (Thermo Fischer Scientific, USA) for selective vagus nerve staining. The animals were allowed to recover for 4 weeks before the *in vivo* imaging experiments. Kupffer cells were labeled *in vivo* by using a single dose of anti-F4/80 conjugated with phycoerythrin (4 mg, clone BM8, eBiosciences, USA).

To investigate the vagus nerve efferent innervation in the liver, we used the ChAT-Td-Tomato transgenic mice. Liver samples were fixed in 4% paraformaldehyde, embedded freezing medium and sectioned using a cryostat. Kupffer cells were immunostained to visualize the neuro-immune interaction. The slides were incubated overnight with the primary rat anti-F4/80 antibody (1:250 dilution, Abcam, USA), washed in phosphate buffered saline and then incubated with the secondary donkey anti-rat Alexa-405 antibody for 1 h (1:250 dilution, Abcam, USA).

2.8. CLP model of sub lethal sepsis

Sepsis was induced by cecal ligation and perforation (CLP). Mice were anesthetized, abdomen was shaved and disinfected and a midline laparotomy was performed. Cecum was exposed, ligated bellow the ileocecal junction and perforated once with a 26G needle and gently squeezed to extrude a small amount of feces from the perforation sites to induce moderate sepsis (Ferreira et al., 2017; Walley et al., 1996). Mice from the VNS group were submitted to the stimulation protocol immediately after CLP. All animals were euthanized after 6 h of sepsis induction for tissue sampling.

2.9. qRT-PCR

Total RNA was extract by using the Aurum total RNA mini kit and reverse transcribed by using iScript cDNA synthesis kit (BioRad Laboratories, USA). For qRT-PCR reactions, we used the iTaq Universal SYBR Green Supermix (BioRad Laboratories, USA) and the 7500 real-time PCR system (Applied Biosystems, USA). Each transcript level was normalized to Gapdh or S26. VNS or Sepsis + VNS groups were used as references for data normalization in Control *versus* VNS and sepsis experiments, respectively. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Supplementary Table 1.

2.10. Cortiscosterone measurement by enzyme-linked immunosorbent assay (ELISA)

Circulating corsticosterone was assessed in serum using a commercially available kit (Cayman Corticosterone ELISA kit, USA) according to manufacturer.

2.11. Laser Doppler blood flow assessment

For peripheral blood flow assessment, anesthetized mice were submitted to the surgical procedures for VNS. After vagus nerve isolation, the animals were placed on a high-resolution laser Doppler imaging system (moorLDI2-HIR; Moor Instruments, UK). The caudal vein blood flow was monitored before (baseline), immediately after VNS and 5 min after stimulation.

2.12. Liver injury

To investigate whether VNS results in liver injury, we monitored the levels of alanine aminotransferase (ALT) activity in serum of unstimulated or stimulated mice by using a kinetic Transaminase ALT (TGP) kit (Bioclin, Brazil).

2.13. Statistical analysis

All data were tested for normal distribution. Differences between two samples were analyzed for significance using unpaired two-tailed Student's *t* test. Differences between three or more groups were analyzed using One-way ANOVA with Newman-Keuls Multiple Comparison Test (for Gaussian distributions) or Kruskal-Wallis test with Dunn's Multiple Comparison Test (for non Gaussian distributions). Values are from multiple biological replicates within an experiment and reported as the mean ± s.e.m.

3. Results

3.1. Vagus nerve stimulation increases phagocytosis by Kupffer cells without acute changes in cytokine production

To investigate the effect of vagus nerve circuit on the immunological function of the liver, we submitted mice to electrical stimulation of the vagus nerve (VNS). Following stimulation, Kupffer cells presented more surface projections when compared to unstimulated cells, which was suggestive of macrophage activation (Fig. 1B). Therefore, we addressed liver macrophage phagocytic function in real time using *in vivo* imaging. For this purpose, we immunostained Kupffer cells with F4/80 antibody and injected *E. coli*^{gfp} intravenously to monitor the dynamics of phagocytosis by confocal microscopy (Fig. 1A). Cervical VNS increased the ability of Kupffer cells to phagocytize bacteria when compared to control mice (Fig. 1B–D), which was also observed when the stimulation was performed on the ventral (or anterior) abdominal trunk of the vagus nerve (AbVNS, Fig. 1E). This response was abolished when we stimulated the proximal end (segment above the sectioning) of the vagus nerve from vagotomized mice (VNX). Similar results were obtained when we performed abdominal vagotomy (AbVNX) (Fig. 1B–E, Supplementary videos 1–3). Together, our results suggest that a vagus-vagal reflex was involved in the regulation of Kupffer cell activity. We further tested our hypothesis injecting ^{99m}Tc-labelled colloidal tin intravenously in mice, a radiopharmaceutical commonly used to evaluate the phagocytic activity by Kupffer cells (Shim et al., 2009). Corroborating our findings, ^{99m}Tc-colloidal-tin uptake by the liver was also increased following VNS (Fig. 1F). Surprisingly, stimulation of the vagus nerve did not impact the uptake of the radiopharmaceutical by the lungs and spleen, other organs with a well-developed reticuloendothelial system, suggesting that this response may be specific to the liver (Fig. 1G).

VNS regulation of phagocytosis activity was not paralleled by alterations in hepatic mRNA levels of cytokines as measured immediately after the ending of the *in vivo* assays (Fig. 2). In addition, the results were not dependent on the number of Kupffer cells or peripheral blood flow, nor caused liver injury (Supplementary Fig. 1A–D).

3.2. Neuro-immune interaction between vagus nerve and Kupffer cells

Next, we investigated the innervation of liver parenchyma by the vagus nerve. We first used a selective staining using the tracer DiO. We showed proximity between vagal fibers and some Kupffer cells within the sinusoids (Fig. 3A). We then used a fluorescent reporter ChAT line (ChAT-TdTomato mice) to investigate the presence of cholinergic fibers within the liver. This is a transgenic mouse model in which cells expressing Choline-O-Acetyltransferase (ChAT) also express a red fluorescent protein (TdTomato). By using this approach, we identified ChAT-positive fibers running along the sinusoids adjacent to Kupffer Cells, which also expressed ChAT (Fig. 3B).

The increase in ChAT expression in immune cells has been shown after vagus nerve activation, leading to increased acetylcholine synthesis and the control of immune responses (Rosas-Ballina et al., 2011). Here, we isolated Kupffer cells from the livers of animals after VNS and observed an increase in ChAT mRNA levels in the resident liver macrophages obtained from vagus-nerve stimulated mice when compared to controls (Fig. 3C). Taken together, our results suggest the presence of vagal fibers in the liver parenchyma, including fibers from the efferent cholinergic arm of the vagus that can modulate macrophage responses.

3.3. Cholinergic signaling mediates vagus nerve regulation of phagocytosis by Kupffer cells

These *in vivo* results prompted us to study the role of cholinergic

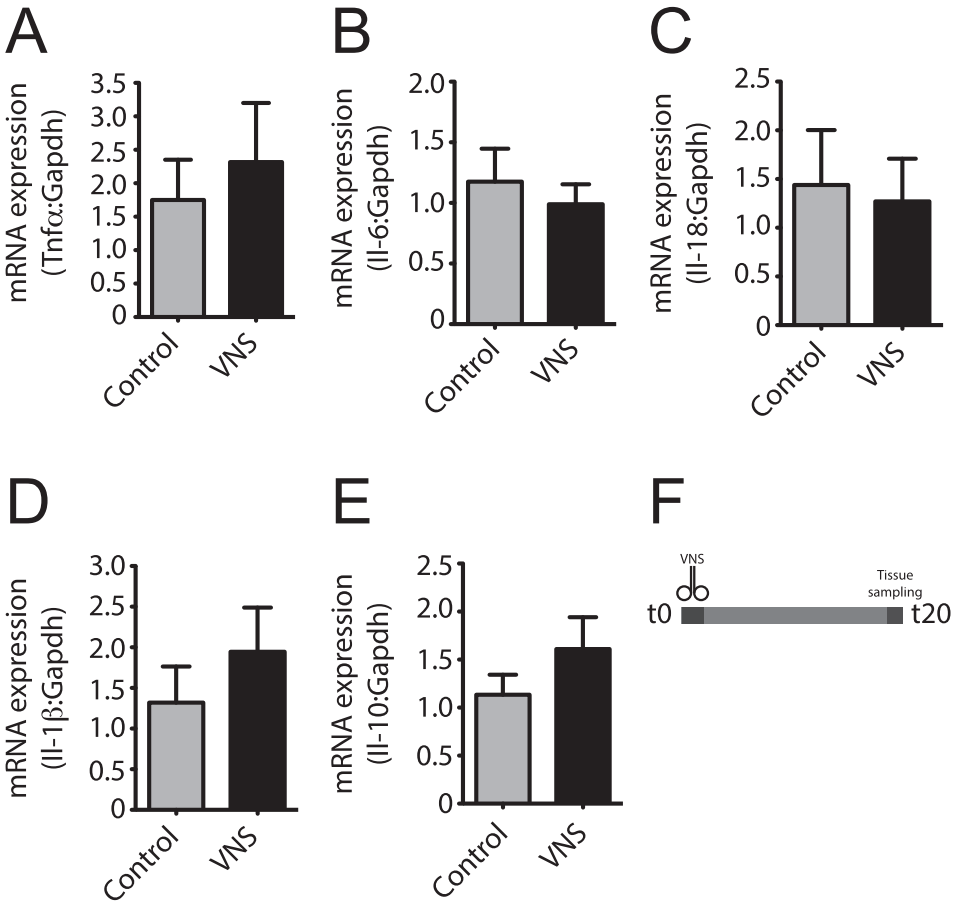

Fig. 2. Expression of pro- and anti-inflammatory cytokines in the liver after vagus nerve stimulation (VNS). Livers from mice submitted VNS were collected after the ending of the phagocytosis assays. (A) TNFa, (B) Il-6, (C) Il-18, (D) Il-1b and (E) Il-10 mRNA expression (n = 5/group; error bars = mean ± s.e.m). (F) Experimental timeline. t0 = begin of experiment; t20 = total duration of the experiment in minutes.

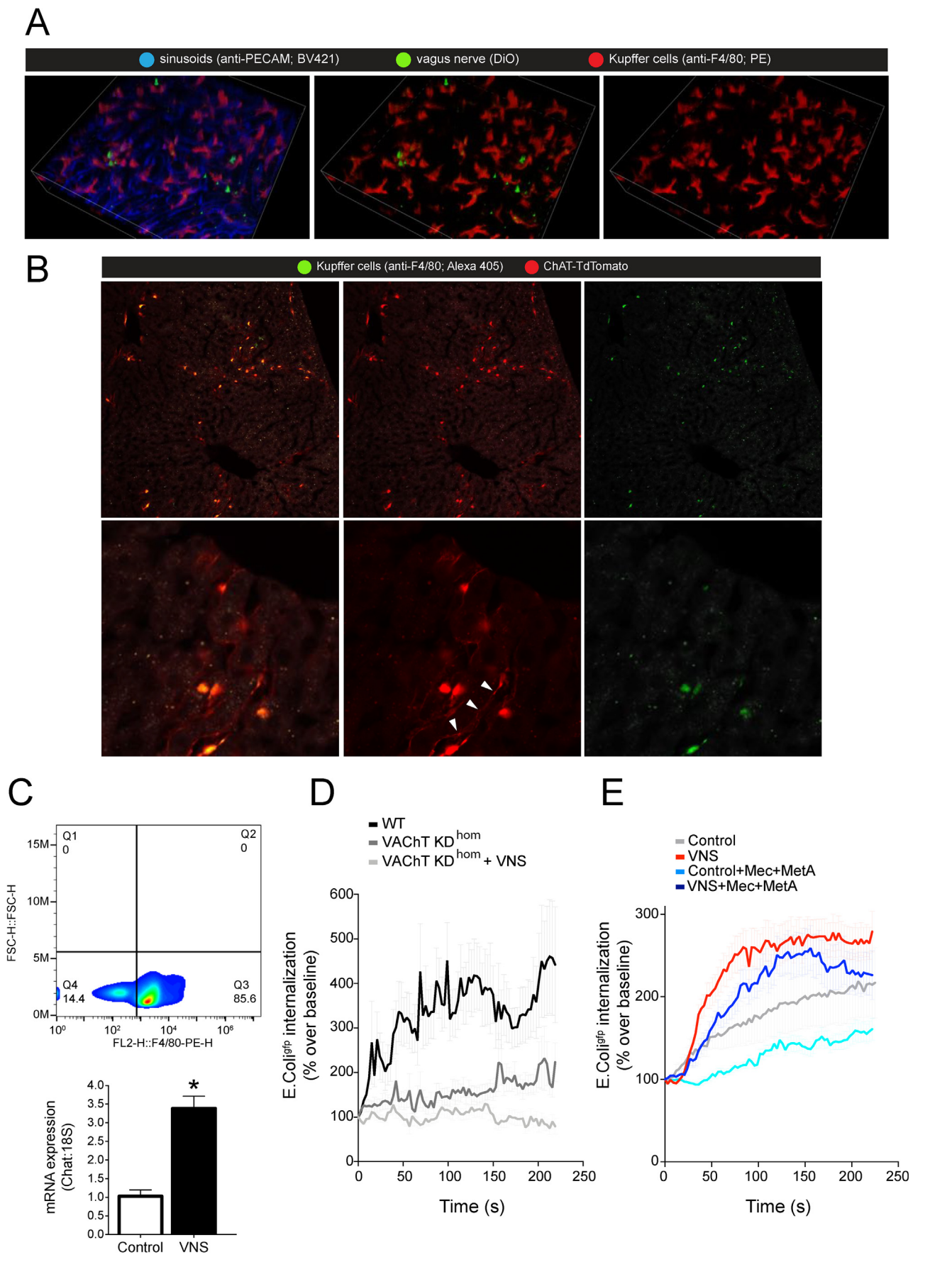

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Fig. 3. Neuro-immune interaction between vagus nerve and Kupffer cells. (A) *In vivo* 3D reconstruction from Z-stack images of the liver following selective vagus-nerve staining using DiO neurotracer (green). Kupffer cells were immunostained with anti-F4/80-PE antibody (red). Sinusoids were stained using anti-PECAM-BV421 antibody (blue). (B) ChAT-TdTomato (red) positive fibers (arrowheads) within liver parenchyma and in close contact with Kupffer Cells (green). (C) ChAT expression in isolated Kupffer cells (F4/80 positive cells, Q3 in the cytometry plot) from control and VNS livers (*P < 0.05). (D–E) Time course of phagocytosis dynamics by Kupffer cells after cholinergic signaling impairment in transgenic VAChT-KD^{hom} mice (n = 3 mice/group) (D) or pharmacological blockade of acetylcholine receptors (n = 4 mice/group) (E). Data is presented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

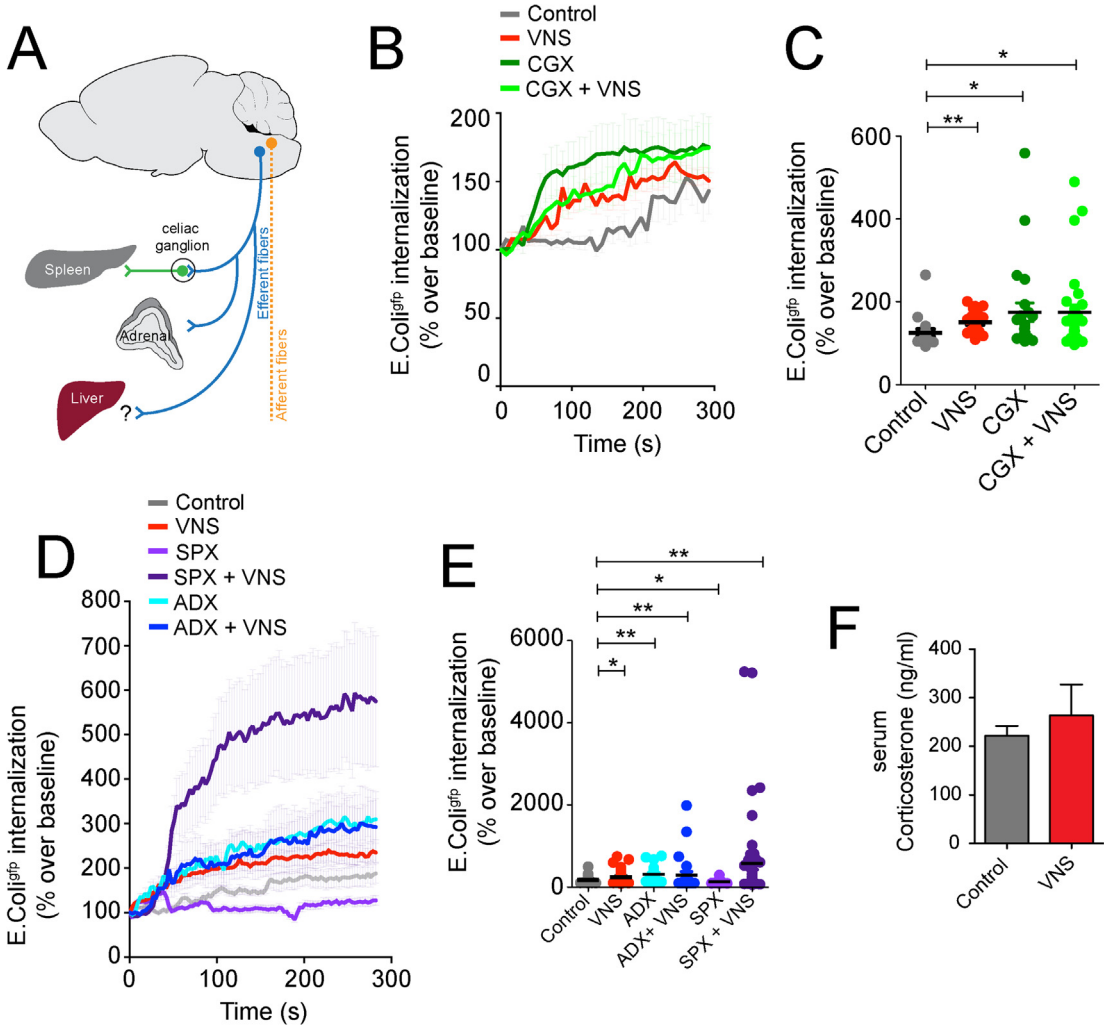

Fig. 4. Cholinergic signaling mediates the regulation of Kupffer cells activity. (A-B) Time course of phagocytosis dynamics by Kupffer cells after cholinergic signaling impairment in transgenic VAChT-KD^{hom} mice (A) or pharmacological blockade of acetylcholine receptors (B). (C-G) Phagocytosis activity by Kupffer cells after celiac ganglion removal (CGX), splenectomy (SPX), or bilateral adrenalectomy (ADX) (n = 50 cells/group; 3 mice/group; **P < 0.01; *P < 0.05; One-way ANOVA with Newman-Keuls Multiple Comparison Test; error bars = mean ± s.e.m). (H) Serum corticosterone concentrations (n = 4/group; error bars = mean ± s.e.m). VNS = vagus nerve stimulation.

signaling on the modulation of phagocytosis by Kupffer cells. Acetylcholine is the main neurotransmitter released by vagus nerve efferent fibers involved in the control of immune responses. First, we used the homozygous vesicular acetylcholine transporter knock down mice (VAChT-KD^{hom}), which have a reduction between 60% and 70% in VAChT expression and decreased acetylcholine release (Prado et al., 2006). Kupffer cells from VAChT-KD^{hom} mice presented a remarkable reduction in the ability to phagocytize bacteria when compared to WT cells, which was not increased following VNS (Fig. 3D). Accordingly, the pharmacological antagonism of nicotinic and muscarinic acetylcholine receptors resulted in reduced capacity of basal and VNS-induced phagocytosis by Kupffer cells (Fig. 3E). Together, these results point to an important role of cholinergic signaling on the vagal-mediated modulation of Kupffer cell activity.

Vagal control of the immune system may involve complex neural pathways. Vagus nerve may stimulate the splenic nerve at the celiac ganglion and control the production of pro-inflammatory cytokine by macrophages in the spleen (Rosas-Ballina et al., 2011) or, in addition, can affect the adrenal glands and stimulate the secretion of dopamine or corticosterone to promote anti-inflammatory responses (Pavlov et al., 2003; Torres-Rosas et al., 2014). Thus, we investigated whether VNS had direct effects on the liver or were due to the activation of any of these circuits (Fig. 4A). After surgical removal of the celiac ganglion and adrenal glands, we observed an increase in the phagocytosis of *E. coli*^{gfp} by Kupffer cells in the liver, which was not further stimulated by VNS (Fig. 4B-E). On the other hand, splenectomy (SPX) caused a negative impact on phagocytosis. Interestingly, we observed increased Kupffer cell phagocytosis in splenectomized (SPX) mice submitted to

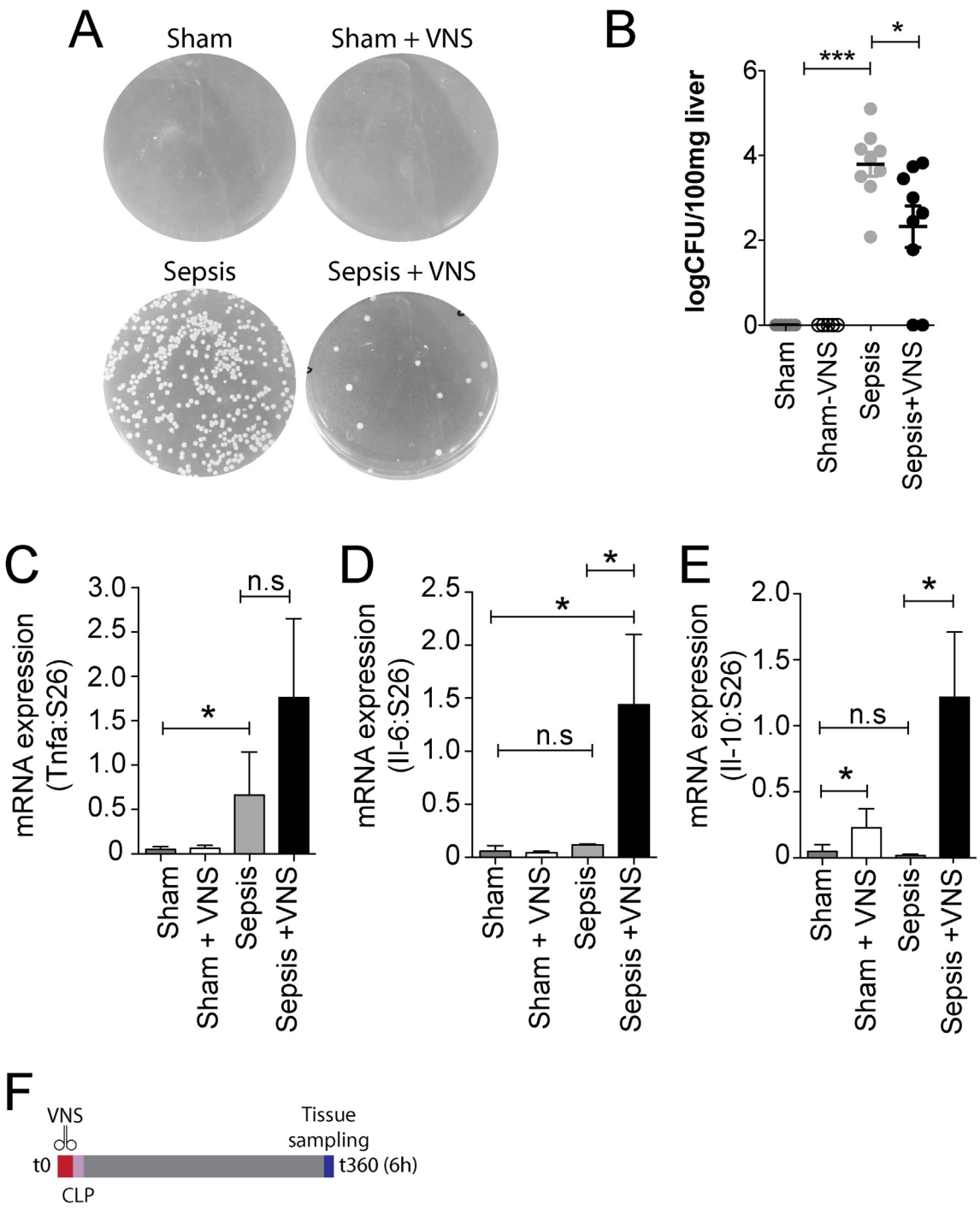

Fig. 5. Vagus nerve stimulation (VNS) promotes bacterial clearance, pro-regenerative and anti-inflammatory phenotypes in the liver. (A) Representative images of bacterial growth in CLP-induced sepsis. (B) Quantification of the colony formatting units (CFU) (n = 9/group; ***$P < 0.001$, *$P < 0.05$; One-way ANOVA with Newman-Keuls Multiple Comparison Test; error bars = mean ± s.e.m). (C–E) Expression of Tnfα, Il-6 and Il-10 in the liver during sepsis (n = 5; * $P < 0.05$; Kruskal-Wallis test with Dunn's Multiple Comparison Test; error bars = mean ± s.e.m). (F) Experimental timeline. t0 = begin of experiment; t360 = total duration of the experiment in minutes.

VNS; however, in a higher magnitude than in the VNS alone group (Fig. 4D, E). No differences were found in circulating corticosterone concentrations (Fig. 4F). Our data suggest that the celiac ganglion, spleen and adrenal glands can partially modulate the phagocytic activity of Kupffer cells in the liver, although the basis of such relationships are unknown.

3.4. Vagus nerve stimulation increases bacterial removal in the liver during sepsis

Based on our findings showing that vagus nerve regulates phagocytosis activity of Kupffer cells in physiological conditions, we asked whether this circuitry would play any role in hepatic bacterial clearance during a non-lethal septic model induced by cecum ligation and perforation (CLP). CLP promoted significant bacterial load in the liver, as observed by the number of colony formatting units (CFU) (Fig. 5A, B).

VNS significantly reduced hepatic bacterial burden, suggesting that the stimulation of the vagus nerve may prime liver bacterial clearance. Previous studies show that cholinergic signaling provides regulatory signals that reduce the production of pro-inflammatory cytokines (Almeida Amaral et al., 2016; Borovikova et al., 2000; Pavlov and Tracey, 2017). During sepsis, we observed an increase in hepatic Tnfα mRNA levels, whereas the expression of Il-6 and Il-10 were comparable to the sham-operated group (Fig. 5C–E). VNS did not impact in the expression of Tnfα following CLP, but increased the levels of Il-6 and Il-10. Importantly, VNS resulted in the increase of Il-10 production within the liver even in the absence of an immune challenge (Fig. 5E, sham + VNS group); however, in a lower magnitude when compared to septic mice (Fig. 5E, sepsis + VNS group).

4. Discussion

Vagus nerve signaling is a critical component of the cholinergic anti-inflammatory pathway. This neuro-immune reflex is generally considered as a physiological system to control inflammatory responses in different pathologies, including endotoxaemia, sepsis, burn injuries, metabolic syndrome and arthritis (Almeida Amaral et al., 2016; Bassi et al., 2017; Borovikova et al., 2000; Kimura et al., 2016; Lopez et al., 2012). The current accepted mechanism for this reflex involves nAChR activation and a downstream signaling pathway leading to the inhibition of NF-κB p65 activity, which consequently reduces the transcription of pro-inflammatory cytokines (de Jonge et al., 2005; van der Zanden et al., 2009). Despite the cholinergic anti-inflammatory signaling constitutes a rapidly evolving field, the impact of this neuro-immune reflex in the functions of liver macrophages are still poorly understood.

For the first time we imaged the *in vivo* hepatic microenvironment of mice after vagus nerve stimulation (VNS) and showed a neuro-immune interaction that primes Kupffer cells capacity of phagocytosis and to arrest bacteria (*E. coli*) under flow. This reflex was abrogated when we surgically sectioned the left vagus trunk, the major innervation to gastrointestinal organs including the liver (Berthoud et al., 1991) or used animal models of cholinergic deficiency. Our data indicate that a vagovagal reflex dependent on cholinergic tonus is involved in this phenotype. Our hypothesis is corroborated by previous *in vitro* findings showing that cholinergic signaling through nicotinic receptors regulates the subcellular distribution of proteins, such as the Dynamin-2 GTPase, which is involved in phagosome formation (Gold et al., 1999; Huynh and Grinstein, 2008; van der Zanden et al., 2009). Therefore, it is likely that a similar mechanism relying on the activation of phagocytosis pathways and in the recruitment of proteins important for phagosome assembly are involved in this acute response to vagus nerve activation. VNS also resulted in increased expression of Choline-O-Acetyltransferase (ChAT), a key enzyme in acetylcholine synthesis. Accordingly, increased ChAT levels in immune cells were observed in immune cells within the spleen after the activation of vagus nerve. This lead to higher acetylcholine synthesis which was involved in the control of immune responses (Rosas-Ballina et al., 2011). The similarity of the present findings with those previously published suggest that this maybe a shared mechanism of the cholinergic anti-inflammatory pathway.

Interestingly, the acute modulation of Kupffer cells phagocytic activity was not paralleled by acute alterations in the cytokine expression profile within the hepatic microenvironment, which was only observed 6 h following VNS. It has already been shown that the secretion of cytokines by Kupffer cells can be modulated by the action of acetylcholine through nicotinic receptors during an immune challenge or in pathologies in which a inflammatory response is an important component, including drug-induced liver injury, nonalcoholic steatohepatitis (NASH) and other chronic liver diseases (Hiramoto et al., 2008; Kimura et al., 2016; Li et al., 2014; Metz and Pavlov, 2018; Ni et al., 2016). Taken together, our data suggests a dual effect of vagus nerve circuitry within the liver that can be evoked even in the absence of an immune challenge: (1) a fast reaction that optimizes phagocytosis and (2) a long-term response that modulates cytokine production. This can be viewed as an advantage in the fine-tuning and balancing of immune responses to immunogenic threats.

The liver is considered as one of the first lines of defense against blood-borne infections owning to its unique anatomical location and cellular characteristics. First, it has a dual blood supply, receiving the blood drained directly from the gastrointestinal tract through the hepatic portal vein and also from the hepatic artery. Second, because the hepatic microenvironment harbors the largest population of resident macrophages (Kupffers cells), natural killer (NK) cells and natural killer T (NKT) cells when compared to other organs in the organism (Jenne & Kubes, 2013; Balmer et al., 2014). Also, other immune cells as lymphocytes, dendritic cells, monocytes and granulocytes are abundantly found within the liver (Nakagaki et al., 2018). Therefore, the liver screens the blood in the gastrointestinal tract and in the systemic circulation and serves as a highly efficient filter against antigens, toxins and microorganisms that translocate from the guts (Broadley et al., 2016; Strnad et al., 2017). In the present study, we showed that the increase in Kupffer cells phagocytic activity mediated by the vagus nerve primes the bacterial removal within the liver in an experimental model of sepsis. Moreover, the vagal circuitry also differentially modulates the production of Il-6 and Il-10, with no impact in Tnfα mRNA levels. Il-6 and Tnfα are well known cytokines produced by Kupffer cells that facilitates the transition of quiescent hepatocytes from G_0 to G_1, promoting hepatocyte proliferation and liver regeneration (Cressman et al., 1996; Hayashi et al., 2005; Kovalovich et al., 2000; Yamada et al., 1997), whereas Il-10 is a key anti-inflammatory mediator (Ouyang et al., 2011). Taken together, our data show that the vagus nerve constitutes an important signaling circuit to facilitate bacterial clearance, promote an anti-inflammatory and a pro-regenerative environment in liver parenchyma.

The vagal efferent arm of the anti-inflammatory cholinergic signaling originates at the dorsal motor nucleus (DMN) of the vagus nerve and projects to the viscera, including the spleen, adrenal glands and the liver (Huston et al., 2006; Rosas-Ballina et al., 2011; Torres-Rosas et al., 2014). Based on our findings reporting the presence of vagal fibers, including efferent (motor) endings, close to Kupffer, we suggest a liver-brain axis that directly modulates hepatic immune responses. As aforementioned, VNS was also capable of increase ChAT expression in Kupffer cells, which straighten the possibility of a liver branch of the anti-inflammatory cholinergic signaling. This interpretation is further corroborated by the results presented here showing that both splenic and adrenal neural branches are not crucial for the onset of the vagal neuro-immune reflex in the liver, although they can partially modulate this response. To some extend, this can be explained by the existence of a shared mechanism that results in the production of humoral factors in organs innervated by the vagus nerve that control systemic immune responses.

Although the distribution of cholinergic nerves within the hepatic parenchyma depends on the species studied, it was previously shown in mice and humans that some acetylcholinesterase-positive fibers innervate the hepatocytes and the hepatic sinusoids, especially in the periportal region (Lautt, 1983; Reilly et al., 1978), which is agreement with the present data.

In summary, our study provides evidences for a new branch of the vagus nerve anti-inflammatory reflex, which regulates phagocytosis activity by Kupffer cells, increases bacterial removal by the liver and promotes a pro-regenerative and anti-inflammatory phenotype.

Declaration of Competing Interest

Authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.06.041.

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