Video Article

Increased Recovery Time and Decreased LPS Administration to Study the Vagus Nerve Stimulation Mechanisms in Limited Inflammatory Responses

Erwan Le Maître[1], Priya Revathikumar[1], Johanna Estelius[1], Jon Lampa[1]

[1]Department of Medicine, Unit of Rheumatology, Center for Molecular Medicine (CMM), Karolinska Institute, Karolinska University Hospital

Correspondence to: Erwan Le Maître at erwan.lemaitre@ki.se

URL: https://www.jove.com/video/54890
DOI: doi:10.3791/54890

Keywords: Immunology, Issue 121, Vagus nerve, electrical stimulation, cholinergic anti-inflammatory pathway, inflammation, neuro-immune communication, cytokines

Date Published: 3/29/2017

Citation: Le Maître, E., Revathikumar, P., Estelius, J., Lampa, J. Increased Recovery Time and Decreased LPS Administration to Study the Vagus Nerve Stimulation Mechanisms in Limited Inflammatory Responses. *J. Vis. Exp.* (121), e54890, doi:10.3791/54890 (2017).

Abstract

Inflammation is a local response to infection and tissue damage mediated by activated macrophages, monocytes, and other immune cells that release cytokines and other mediators of inflammation. For a long time, humoral and cellular mechanisms have been studied for their role in regulating the immune response, but recent advances in the field of immunology and neuroscience have also unraveled specific neural mechanisms with interesting therapeutic potential. The so-called cholinergic anti-inflammatory pathway (CAP) has been described to control innate immune responses and inflammation in a very potent manner. In the early 2000s, Tracey and collaborators developed a technique that stimulates the vagus nerve and mimics the effect of the pathway. The methodology is based on the electrical stimulation of the vagus nerve at low voltage and frequency, in order to avoid any side effects of overstimulation, such as deregulation of heart rate variability. Electrical devices for stimulation are now available, making it easy to set up the methodology in the laboratory. The goal of this research was to investigate the potential involvement of prostaglandins in the CAP. Unfortunately, based on earlier attempts, we failed to use the original protocol, as the induced inflammatory response either was too high or was not suitable for enzymatic metabolism properties. The different settings of the original surgery protocol remained mostly unchanged, but the conditions regarding inflammatory induction and the time point before sacrifice were improved to fit our purposes (*i.e.,* to investigate the involvement of the CAP in more limited inflammatory responses).

The modified version of the original protocol, presented here, includes a longer time range between vagus nerve stimulation and analysis, which is associated with a lower induction of inflammatory responses. Additionally, while decreasing the level of lipopolysaccharides (LPS) to inject, we also came across new observations regarding mechanistic properties in the spleen.

Video Link

The video component of this article can be found at https://www.jove.com/video/54890/

Introduction

Innate immunity provides an immediate first line of defense against infections and diseases in a wide range of organisms. It not only initiates the primary immune response to eliminate the threat, but it also plays a pivotal role in activating and educating the adaptive immunity that carries out secondary immune responses in a pathogen-specific manner. Inflammation is orchestrated by a plethora of cytokines and chemokines, which in turn have the ability to attract other immune cells to the site of infection and to induce the cardinal signs of inflammation, such as redness, swelling, pain, loss of function, and fever. The duration and intensity of inflammation depend on several factors, but resolving the inflammation and restoring homeostasis is a critical step to avoid the onset of chronic inflammatory diseases. Recent advances in the field of neuroscience and immunology have unraveled specific neural mechanisms with immense therapeutic potential to control inflammation both in the central nervous system and in the periphery. One of these mechanisms is the cholinergic anti-inflammatory pathway (CAP), also known as the inflammatory reflex, which is driven by the autonomic nervous system[4,5].

It is currently thought that inflammatory mediators activate sensory nerves and send signals concerning the state of inflammation to the central nervous system. A reflex response is then activated through the efferent vagus nerve. An extensive study on the anatomical details of the CAP has revealed a parasympathetic-sympathetic model composed of two nerves, the vagus nerve and splenic nerve, respectively[6]. In the CAP, the activated cholinergic efferent vagus nerve ends in the celiac-mesenteric ganglion, resulting in the activation of the adrenergic splenic nerve by a mechanism yet to be explored. The splenic nerve, thus activated, is known to innervate in close proximity to immune cells in the white pulp, marginal zone, and red pulp of the spleen, the principal and mandatory organ of the CAP[7,8]. Norepinephrine (NE) from the splenic nerve endings binds to the corresponding β_2 adrenergic receptors expressed on splenic T lymphocytes. This induces choline acetyl transferase (ChAT)-mediated acetylcholine (ACh) release, which in turn activates α7 nicotinic acetylcholine receptors (α7nACh) on macrophages, thereby limiting cytokine production and inflammation[2]. Consequently, it is now clear that the nervous system is able to regulate inflammation in peripheral tissues and to restore local immune homeostasis.

As the name of the pathway suggests, the ACh system is of central importance to the functioning of this neuro-immune regulating pathway. Interestingly, the mechanisms involved in the activation of the CAP seem to be different in the periphery and in the central nervous system. While the importance of nicotinic receptors (α7nAChR) in the spleen has been demonstrated earlier[9], muscarinic receptors (mAChR) are mandatory for the central activation of the pathway[10,11]. More recently, peripheral administration of a centrally-acting M1 muscarinic agonist significantly suppressed serum and spleen tumor necrosis factor α (TNFα) during lethal murine endotoxemia, an action that required intact vagus nerve and splenic nerve signaling[12]. We have also shown recently that mice lacking prostaglandin E_2 (PGE_2) were not able to respond to vagus nerve stimulation and did not down-regulate the LPS-induced release of cytokines in the serum and spleen[3]. Therefore, the CAP might also be regulated by systems other than the main ACh pathway.

The vagus nerve has been named as such because of its wandering course in the body, innervating principal organs including the liver, lung, spleen, kidneys, and gut[13]. Considering this large innervation and the very potent immunosuppressive effect of the vagus nerve, the therapeutic potential of the CAP could cover a wide range of inflammatory conditions. The vagus nerve can be electrically (or mechanically) activated, with control over the voltage and frequency, and contrary to conventional treatment, with no drugs added to the body. Trials are currently underway in rheumatic patients, for instance, to test the clinical significance of VNS in treating chronic inflammation[14]. Altogether, the neuro-immune communication and regulation of inflammation are currently under investigation, which will provide a possible alternative treatment to conventional therapy. Therefore, analysis of the vagus nerve stimulation effect in the different innervated organs, but also characterization of the potential therapeutic action in animal models of chronic inflammation, would definitely give insights and hope for new potential therapeutic targets.

The original methodology developed by Tracey and colleagues[4] could not be transposed to our field of research due to overstimulation of the inflammatory response (by a lethal dose of LPS) and a too-short time range between CAP activation and the read-out. In the present paper, we will present the changes made to the original protocol, compare the two different methodologies on cytokine levels, and highlight a new and opposite observation on the target organ (the spleen).

Protocol

All animal experiments were performed according to the guidelines for the care and use of animals approved by the local Ethics Committee at Karolinska Institutet, Stockholm. The local Ethics Committee follows the European Union directives on animal care.

NOTE: The main changes from the original protocol are the recovery time after surgery (6 hr *versus* 1 hr) and the level of LPS injected (2 mg/kg *versus* 15 mg/kg). Otherwise, the different settings related to the surgery itself have not been changed.

1. Preparing Material for Stimulation

1. Turn on the computer and the data acquisition system linked to the stimulating electrode (**Figure 1A**).
2. Enter the Acknowledge program.
3. Prepare a stock solution of LPS at a concentration of 5 mg/ml in 1x PBS, aliquot it, and store it at -20 °C. On the day of the experiment, thaw an aliquot and prepare an adequate sample of LPS (0.5 mg/ml) in order to inject around 100 µl into the animal, according to the weight.

2. Preparing the Animal for Anesthesia

1. Use C57Bl/6 mice. Maintain them under climate-controlled conditions with a 12-hr light/dark cycle, feed them standard rodent chow, and give them water *ad libitum*.
2. Perform surgery on mice that weigh around 25 g on the day of the experiment.
   NOTE: When inflammatory reactions are induced, it is particularly important to perform regular checkups and observations of the clinical reactions in the animals. Premature euthanasia by CO_2 inhalation is needed if the animal condition does not fulfill the ethical criteria.
3. Set the anesthetic machine.
   1. Make sure that the tubing is properly connected and is not damaged in any way. Make sure that the ventilated area is working properly. Connect the key filter to the isoflurane bottle and fill the vaporizer with a sufficient amount of isoflurane.
   2. Open the gas supply (air and oxygen) and make sure that the bottles contain enough gas for the experiment. A three-way connector is then able to send the isoflurane flow to the induction chamber or the mask.
4. Turn the three-way connector to the induction chamber. Pick one mouse from the home cage and insert the animal into the chamber. Adjust the flow regulator to 1.0 L/min oxygen and 1.0 L/min air. Adjust the isoflurane concentration to 4 - 5%.
5. When the desired level of anesthesia is reached, When the desired level of anesthesia is reached, shave the surgical area and move the animal from the chamber to the mask. Turn the three-way connector to the mask flow. Adjust the flow regulator to 0.25 L/min oxygen and 0.25 L/min air.
   1. Adjust the isoflurane concentration to 1.5 - 2.5%. Check the level of anesthesia by reflex control and respiratory rate before starting the surgical procedure.
6. Fix the legs of the mouse to the work bench using adhesive tape. Make sure that the nose of the animal is still carefully positioned in the mask.

3. Surgery and Stimulation of the Vagus Nerve

1. Disinfect the surgical area with 70% ethanol.
2. Using a scalpel, carefully incise the skin at the level of the neck (incision of around 1 to 1.5 cm).

Note: In the protocol, the surgery procedure ends here for SHAM-operated animals. Indeed, it has been shown that just touching the nerve with a metal tool already stimulates it to some extent. Therefore, to get a more accurate surgery control animal when using a small amount of LPS, stop the surgery at this step.

3. With the help of a microscope (12.5X objective), isolate the left vagus nerve from the carotid artery using dissecting forceps. First locate the sternocleidomastoid muscle by removing skin and fat layers, and then retract it in order to put the forceps behind both the nerve and the artery.
   Note: The following step is very tricky, as the nerve and artery are closely adhered to each other. Therefore, it is very easy to cut the vessel and kill the animal. However, by placing the forceps very carefully between the nerve and artery, they eventually separate, and it is possible to isolate the nerve.
4. Place the electrode (**Figure 1B**) under the vagus nerve. The needle electrode is quite long, so even if the nerve moves slightly during the stimulation, it will always be in contact with the electrode.
5. Perform an intraperitoneal (i.p.) injection of LPS (2 mg/kg) with the help of a syringe (for the read-out on cytokine levels; *i.e.,* to measure the down-regulating effect).
6. Wait 5 min before starting the stimulation.
7. Stimulate the vagus nerve for 5 min at 5 V and 1 Hz by pushing the start button in the Acknowledge program.
8. Remove the electrode and suture the wound of the animal with surgical suture thread.
9. Spray a No Sting Barrier Film (NSBF) on the wound in order to improve healing and to protect from infections.

4. Recovery of the Animal

1. After surgery, move the animal back to its home cage for awakening and recovery. Under infra-red light in order to maintain body temperature, make sure to monitor the animal until full consciousness has been regained.
2. Let the animal recover in its cage for 6 hr before sacrifice for analysis.
   Note: The effect of the vagus nerve stimulation is very fast and has also been shown to be long lasting (up to 48 hr), so the recovery time can be set by the experimenter according to the needs of the study.

5. Sacrifice for Further Analysis

1. Place the animal in a cage linked to a CO_2 administration device.
2. Set the device on a 5-min cycle of CO_2 inhalation.
3. When the euthanasia is done, collect the organs of interest and directly freeze them on dry ice for further analysis (*e.g.,* the measurement of cytokine levels in spleen extracts using a mouse TH1/TH2 9-Plex assay)[3].

Representative Results

Level of TNFα and Interleukin-1β (IL-1β) after Increasing the Time Lapse after Surgery and Decreasing the Dose of LPS

As shown previously, using the original protocol, VNS decreased the levels of TNFα (169.3 ± 24.9 pg/mg in SHAM versus 39.7 ± 10.8 pg/mg in VNS, $p < 0.001$) and IL-1β (360.0 ± 40.21 pg/mg in SHAM versus 191.7 ± 27.2 pg/mg in VNS, $p < 0.01$) in the spleen after intraperitoneal LPS injection (15 mg/kg) (**Figure 2A**). After changing the time for analysis from 1 to 6 hr and decreasing the LPS dose from 15 to 2 mg/kg, we only observed an effect of VNS on TNFα (13.67 ± 1.81 pg/mg in SHAM versus 8.82 ± 1.20 pg/mg in VNS, $p < 0.05$), while IL-1β was not affected (368.62 ± 35.65 pg/mg in SHAM versus 304.99 ± 43.54 pg/mg) (**Figure 2B**). Without any LPS injection (*i.e.,* saline), no difference could be detected after VNS (**Figure 2C**).

Levels of Keratinocyte Chemoattractant/Human Growth-regulated Oncogen (KC/GRO) in the Spleen after VNS Using the Different Conditions

With the original protocol, we showed that KC/GRO was strongly down-regulated by VNS (597.4 ± 17.8 pg/mg in SHAM versus 416.4 ± 29.7 pg/mg in VNS, $p < 0.001$) (**Figure 3A**). Using a 6-hr time point and 2 mg/kg of LPS, no change was observed between SHAM and VNS animals (**Figure 3B**), while VNS is still potent on TNFα, for instance. However, when no LPS was injected for the 6-hr time point, we observed a strong up-regulation of KC/GRO following VNS (59.37 ± 4.29 pg/mg in SHAM versus 141.22 ± 10.56 pg/mg in VNS, $p < 0.001$) (**Figure 3C**). As expected, most of the other cytokines (TNFα, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, and IFNγ) were at very low levels (if detectable), and no difference could be seen between SHAM and VNS-stimulated animals.

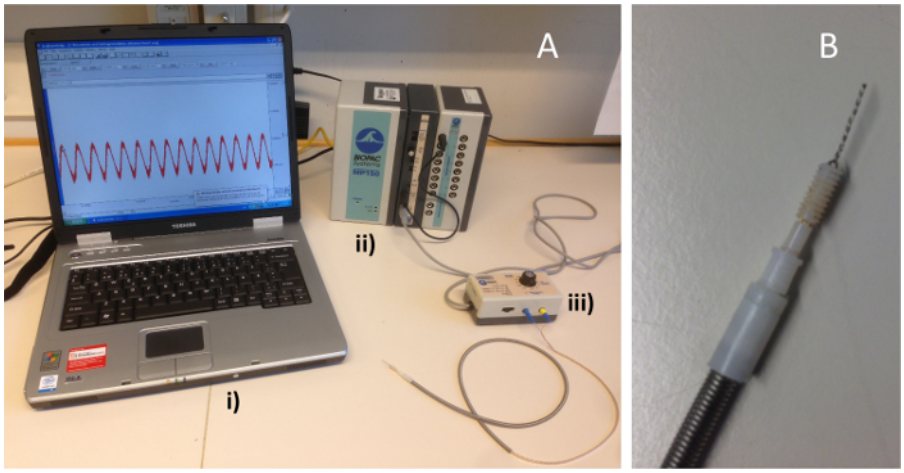

Figure 1: Pictures of the Material Needed in order to Perform the Vagus Nerve Stimulation. A) i) Computer, ii) data acquisition system, and iii) stimulating electrode device. **B)** Close-up of the electrode that is placed under the vagus nerve. Please click here to view a larger version of this figure.

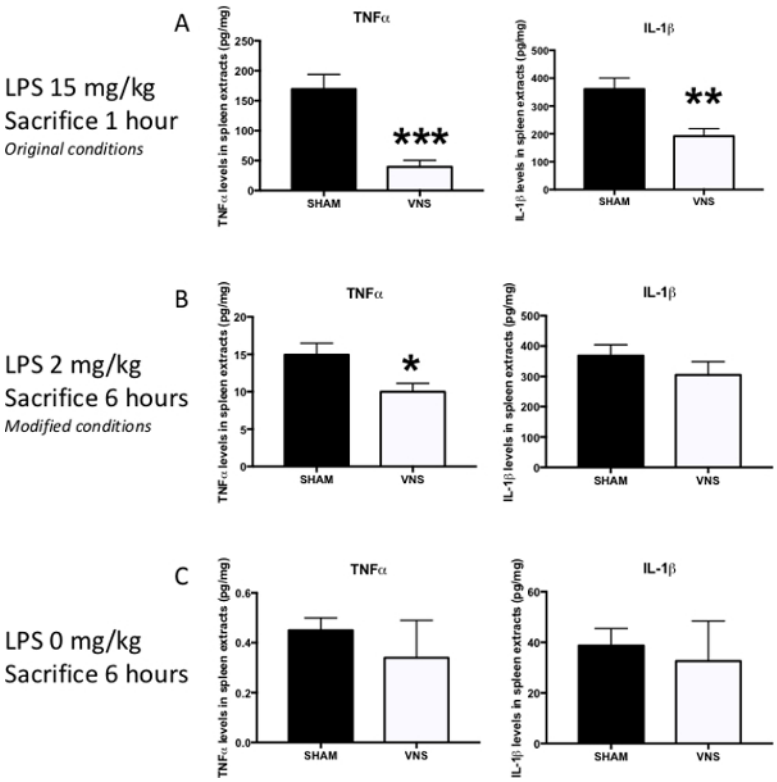

Figure 2: Levels of TNFα and IL-1β in Spleen Extracts of SHAM and VNS-treated Animals. Animals are subjected to SHAM or VNS surgery, according to the following conditions: **A)** 1-hr recovery after i.p. injection of 15 mg/kg LPS, **B)** 6-hr recovery after i.p. injection of 2 mg/kg LPS, and **C)** 6-hr recovery without LPS injection. Levels are measured on a pro-inflammatory cytokine-coated plate and expressed as pg/mg of tissue. Data are expressed as the mean ± SEM. An asterisk shows statistical differences between VNS and SHAM mice (n = 8 in each group). Student's *t*-test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Please click here to view a larger version of this figure.

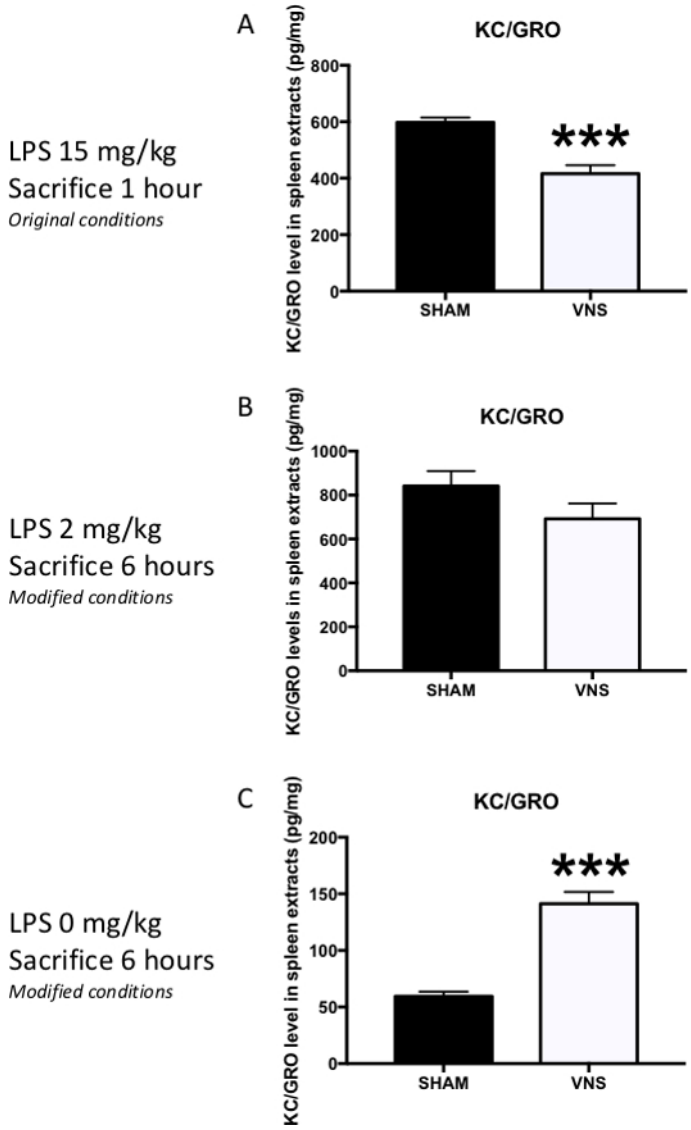

Figure 3: Levels of KC/GRO in Spleen Extracts of SHAM and VNS-treated Naïve Mice. Animals are subjected to SHAM or VNS surgery, according to the following conditions: **A)** 1-hr recovery after i.p. injection of 15 mg/kg LPS, **B)** 6-hr recovery after i.p. injection of 2 mg/kg LPS, and **C)** 6-hr recovery without LPS injection. Levels are measured on a pro-inflammatory cytokine-coated plate and expressed as pg/mg of tissue. Data are expressed as the mean ± SEM. An asterisk shows statistical differences between VNS and SHAM mice (n = 8 in each group). Student's *t*-test *** p <0.001.

Discussion

Since its discovery in the early 2000s, the mechanisms of the CAP have been thoroughly studied. We now have a good picture of the pathway, and in particular, the target organ, the spleen, where NE, memory T cells, Ach, and macrophages work as a very efficient team to down-regulate inflammatory mediators[2]. We have also recently published data on the importance of a functional prostaglandin system in mice, in particular, PGE_2, which is obviously a mandatory component for ACh release in the spleen after activation of the CAP[3].

The experimental technique, known as vagus nerve stimulation, can easily be performed in a laboratory, but a few important protocols must be observed regarding the animals and the surgery procedure. We use a sine-wave stimulation signal, which is supposed to be a "charge-balanced" stimulation, in order to avoid building up electrical charges in the nerve that could destroy it (as opposed to monophasic stimulation). While stimulating the nerve, it is not possible to separate the afferent from the efferent signal, which can only be done for other purposes with vagotomy experiments. The duration and frequency used for the experiment are known to have an effect on cytokine release following inflammatory challenge, for instance, but would not affect the heart rate or give obvious side effects in healthy controls.

First of all, the experimenter must always keep in mind that the handling, surgery, and recovery of the animals must be done according to the ethical rules for animal care. The other point is that practicing the surgery is of great importance. Isolating the vagus nerve is a difficult step, which can be lethal for the animal if vascular damage occurs. With experience, the surgery can be done within 15 - 20 min, meaning that the animal does not have to be anesthetized for a long period of time, which helps the recovery.

A discussion can also be raised regarding the choice of a good SHAM-operated animal. In our protocol, we used animals that only underwent surgery at the level of the neck, without placing the electrode under the vagus nerve. The reason for choosing this option was that just placing the electrode under the nerve would mechanically stimulate the nerve to some extent (as has been observed previously)[19], creating the risk of masking potential mechanisms that occur at very low levels of inflammation. If using very high doses of LPS, the best SHAM-operated control would probably be to place the electrode under the nerve, as the effect of the SHAM surgery would not interfere or mask the obvious electrical stimulation effect on the burst of cytokines.

We have developed the current protocol to fit the different issues encountered within our field of research. As discussed in our previous paper, we looked at the possible involvement of the prostaglandin system in the CAP. Due to enzymatic metabolism, we thought that the 1-hr recovery time of the original protocol would not suit our experiment. Therefore, we extended the time lapse after surgery to 6 hr. In so doing, we also had to titrate down the dose of LPS in order to meet the ethical criteria. We chose a recovery time of 6 hr, a delay that we thought long enough for this purpose. We also titrated the LPS from 15 mg/kg (the original protocol) down to 0 mg/kg. At 2 mg/kg, we saw the last effect of the VNS on cytokines (**Figure 2**), an effect that disappeared at lower doses.

While the main effect of the VNS in the spleen is known and characterized by the activation of memory T cells and thereafter, macrophages-the cells that allow the decrease of cytokines-we also showed here that VNS seems to directly induce the release of mediators (it is still unclear from which cells, necessitating further study) in order to recruit other cell types from the primary response to inflammation. Indeed, while VNS strongly decreased KC/GRO after a strong inflammatory induction (15 mg/kg LPS; *i.e.,* the original protocol), it activates a very fast release of KC/GRO in the absence of inflammation. The chemokine KC/GRO, a IL-8 related protein with strong chemotactic properties, is known to have an important role in leucocyte development (*e.g.,* driving maturation and activation), trafficking (*e.g.,* attraction and recruitment of neutrophils), and function[15]. For instance, neutrophils are important members of the phagocytic system of the innate immune system. They act as the first line of host defense against invading pathogens, but they are also important mediators of inflammation-induced injury[16]. Interestingly, in our attempt to improve the protocol, we then came across this observation that highlights the direct involvement of other cell types in the spleen in vagus nerve functioning.

All the pioneering work in the field has focused on the spleen, the target organ of the CAP, and on the immunoregulatory effect of the pathway in response to sepsis or peripheral systemic inflammation. Importantly, this work was done using acute stimulation of the vagus nerve, such as the one presented in this paper. This can be used for molecular analysis of specific organs or of the short-term effects seen in functional studies involving chronic animal models of inflammation (24 to 48 hr maximum following the surgery).

However, the wandering course of the vagus nerve also implies that the CAP could be of significant use to treat chronic inflammatory diseases involving the different innervated organs[13]. One of the most-studied organs in this context is the gut, with a focus on inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, but also the postoperative-induced ileus[17]. Moreover, the importance of the CAP regulation in many other inflammatory diseases involving the liver, kidney, and lung is also under investigation[18]. Nevertheless, the current protocol could not be used in this context, as the long-term effect of vagus nerve activation would require repetitive stimulations that can of course only be done with an *in vivo* implanted electrode. For this purpose, the animal should undergo a surgery where stimulating cuff electrodes are placed around the vagus nerve[20]. It is also important to note that the latter technique is much more frequently described in humans or in animals of a larger size. Another way of stimulating the vagus nerve is mechanical, as non-invasive and percutaneous stimulation of the vagus nerve showed an immunosuppressive effect in a murine model of endotoxemia[21]. However, the main concerns of this technique would be the reproducibility of the results and how to ensure that the vagus nerve is stimulated the same way in a long-term evaluation of the animal models. Devices have been developed for humans, such as trans-auricular VNS stimulation or trans-cervical VNS stimulation, that deliver electrical pulses, which are well tolerated by the subjects[21]. They have been used mainly to treat epilepsy and migraines and have showed promising results that could lead to future ambulant and exogenous therapy.

To summarize, particularly because of the extensive innervation of the vagus nerve in the body, the CAP regulation is studied in a wide range of inflammatory diseases in the hope of finding interesting molecular mechanistic properties that could lead to potential future therapeutic targets. Here, we presented an acute method to stimulate the vagus nerve. It allows the study of the CAP in limited inflammatory responses thanks to a moderate inflammatory stimulus combined with a longer time range between the VNS and the analysis (allowing enzymatic metabolism to take place, for instance).

Understanding the molecular mechanisms underlying the vagus nerve stimulation effect, as well as its efficient use in the treatment of inflammatory diseases, is of great importance, particularly from a clinical perspective. Indeed, the advantages of the methodology are broad. Due to the nature of extremely fast nerve signaling, it is fast and effective; an observable effect on cytokine levels, for instance, occurs in less than 1 hr.[4] Also, mechanical, non-invasive, and percutaneous stimulation of the nerve can be used, giving hope for future ambulant and easily-administered therapies. Finally, contrary to regular treatment, vagus nerve stimulation uses an endogenous pathway. Therefore, unlike in all drug therapies, no new agents are introduced into the body, thereby avoiding any potential side effects.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The study was supported by the Swedish Research Council, the Swedish Rheumatism Asociation, Karolinska Institute Foundations, Stockholm County Council, The Wallenberg Foundation, and the GV 80 Years' Foundation for research. The authors would also like to thank Hannah Aucott for proofreading the manuscript.

References

1. Nathan, C. Points of control in inflammation. *Nature.* **420** (6917), 846-852 (2002).
2. Rosas Ballina, M., et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science.* **334** (6052), 98-101 (2011).
3. Le Maître, E., *et al.* Impaired vagus-mediated immunosuppression in microsomal prostaglandin E synthase-1 deficient mice. *Prostaglandins Other Lipid Mediat.* **121** (PtB), 155-162 (2015).
4. Borovikova, L.V., *et al.* Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature.* **405** (6785), 458-462 (2000).
5. Olofsson, P.S., Rosas-Ballina, M., Levine, Y.A., Tracey, K.J. Rethinking inflammation: neural circuits in the regulation of immunity. *Immunol. Rev.* **248** (1), 188-204 (2012).
6. Pavlov, V.A., Tracey, K.J. Neural circuitry and immunity. *Immunol Res.* **63** (1-3), 38-57 (2015).
7. Huston, J.M., *et al.* Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. *J. Exp. Med.* **203** (7), 1623-1628 (2006).
8. Rosas-Ballina, M., *et al.* Splenic nerve is required for cholinergic anti-inflammatory pathway control of TNF in endotoxemia. *Proc. Natl. Acad. Sci. USA.* **105** (31), 11008-11013 (2008).
9. Wang, H., *et al.* Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature.* **421** (6921), 384-388 (2003).
10. Pavlov, V.A., *et al.* Central muscarinic cholinergic regulation of the systemic inflammatory response during endotoxemia. *Proc. Natl. Acad. Sci. USA.*, **103** (13), 5219-23 (2006).
11. Pavlov, V.A., *et al.* Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway. *Brain Behav. Immun.* **23** (1), 41-5 (2009).
12. Rosas-Ballina, M., *et al.* Xanomeline suppresses excessive pro-inflammatory cytokine responses through neural signal-mediated pathways and improves survival in lethal inflammation. *Brain Behav. Immun.* **44**, 19-27 (2014).
13. Bellinger, D.L., Lorton, D., Lubahn, D., Felten, D.L. In *Psychoneuroimmunology.* (eds Ader R., Felten D.L., Cohen N.) (Academic, San Diego), 55 -112 (2001).
14. Andersson, U., Tracey, K.J. A new approach to rheumatoid arthritis: treating inflammation with computerized nerve stimulation. *Cerebrum.* **2012**, 3 (2012).
15. Ono, S.J.,Nakamura, T., Miyazaki, D., Ohbayashi, M., Dawson, M., Toda, M. Chemokines: Roles in leucocyte development, trafficking, and effector function. *J. Allergy Clin. Immunol.* **111** (6), 1185-1199 (2003).
16. Silvestre-Roig, C., Hidalgo, A., Soehnlein, O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. *Blood.* Mar 21 (2016).
17. Matteoli, G., Boeckxstaens, G.E. The vagal innervation of the gut and immune homeostasis. *Gut.* **62**, 1214-1222 (2013).
18. Pereira, M.R., Leite, P.E. The involvement of parasympathetic and sympathetic nerve in the inflammatory reflex. *J. Cell. Physiol.* (2016).
19. Levine, Y.A., *et al.* Neurostimulation of the cholinergic anti-inflammatory pathway ameliorates disease in rat collagen-induced arthritis. *PLoS One.* **9** (8), e104530. (2014).
20. Huston, J.M., *et al.* Transcutaneous vagus nerve stimulation reduces serum high mobility group box 1 levels and improves survival in murine sepsis. *Crit. Care Med.* **35** (12), 2762-2768 (2007).
21. Yuan, H., Silberstein, S.D. Vagus nerve and vagus nerve stimulation, a comprehensive review: Part II. *Headache.* **56** (2),259-266 (2016).

