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UNDERSTANDING INFLAMMATION REQUIRES NEUROSCIENCE

Alessandro Gallina

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Understanding Inflammation Requires Neuroscience

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By

Alessandro Gallina

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Principal Supervisor:
Associate Professor Peder S. Olofsson
Karolinska Institutet
Department of Medicine
Division of Cardiovascular Medicine

Co-supervisor(s):
Assistant Professor Laura Tarnawski
Karolinska Institutet
Department of Medicine
Division of Cardiovascular Medicine

Associate Professor Ana Teixeira
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Biomaterials

Opponent:
Professor Paul Peter Tak
University of Cambridge
Department of Medicine
Division of Musculoskeletal Sciences

Examination Board:
Professor David Engblom
Linköping University
Department of Biomedical and Clinical sciences
Center for Social and Affective Neuroscience

Associate Professor Lisa Westerberg
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Assistant Professor Jon Lampa
Karolinska Institutet
Department of Medicine
Division of Rheumatology

ABSTRACT

Inflammation and its resolution are processes subject to neural regulation[1]. The best-characterized immune-regulating reflex is the "inflammatory reflex", in which the efferent branch of the vagus nerve plays a central role in regulating cytokine-release in the periphery. This neural pathway is fundamental to maintaining host homeostasis and preventing potentially damaging inflammation[1–3]. Hence, this biology has already been exploited in several clinical trials regarding potentially new therapies for chronic inflammatory dieseases[4–6]. Of note, cardiovascular disease (CVD) represents the first cause of death worldwide, and its most common manifestation is atherosclerosis which is an inflammatory disease[7,8]. Little is known about the neural control of inflammation in this pathology. Atherosclerotic plaques are not innervated[9], and neurotransmitter signaling in atherosclerosis has not been investigated. The 1998 Nobel Prize for "nitric oxide (NO) as a signaling molecule in the cardiovascular system" reveals the importance of the neurotransmitter acetylcholine (ACh) for regulation of vascular relaxation[10]. ACh is also a key component of the inflammatory reflex, in which cholinergic signals regulate the course of inflammation. In the inflammatory reflex, ACh is produced by nerves and by acetyl-cholinesterase $(ChAT)^+$ T cell under the control of the nervous system, and interacts with alpha 7 nicotinic acetylcholine receptor subunit (α7nAChR)-expressing macrophages (MΦ)[11].

Most of the current knowledge on the inflammatory reflex was obtained from numerous experiments performed in animals, mice *in primis*. Much is still unclear about the details of neural regulation of inflammation and its resolution, and understanding these mechanisms in detail will require further experimental studies. At the same time, it is also important to translate these findings to human pathophysiology, and investigate whether it may inform the design of therapeutic strategies for treatment of inflammatory diseases. This thesis addresses several aspects of this biology:

In **Project I**, we discovered that human $ChAT^+$ T cells participate in cholinergic regulation of vascular function and are found in blood collected from patients in circulatory distress.

In **Project II**, we found components of neurotransmitter signaling in human atherosclerosis, observed an association between low glutamate-receptor expression and adverse clinical events, and found that glutamate signaling regulates smooth muscle cell phenotypic modulation.

In **Project III**, we describe an effective and simple method to electrically stimulate the cervical vagus nerve in mice for the study of experimental inflammation.

In **Project IV**, we provide evidence that electrical activation of the cervical vagus nerve accelerates inflammation resolution in mice through a cholinergic mechanism that involves synthesis of specialized pro-resolving mediators.

Technological limitations in vagus nerve stimulation methods for mice has hampered mechanistic studies of peripheral nerve activation in chronic diseases. Hence, the understanding of mechanisms of vagus nerve regulation of inflammation in chronic diseases is yet incomplete. To solve this, in **Project V** we used a novel approach and developed non-invasive activation of peripheral nerves using temporally-interfering electrical fields. This technology attempts to address methodological shortcoming of "traditional" electrical vagus nerve stimulation (VNS) and enable studies of VNS in genetic mouse models of chronic inflammatory diseases and beyond.

In summary, this thesis studies aspects of neural signaling in inflammation and reveal new details on glutamatergic and cholinergic signals in inflammation and vascular pathophysiology. The work also contributes new methodology which we postulate will be helpful in further understanding of the neural signals that regulate inflammation and for clinical translation of discoveries in this field.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following studies:

I. Tarnawski L, **Gallina AL**, Kort EJ, Shavva VS, Zhuge Z, Martínez-Enguita D, Weiland M, Caravaca AS, Schmidt S, Wang FH, Färnert A, Weitzberg E, Gustafsson M, Eberhardson M, Hult H, Kehr J, Malin SG, Carlström M, Jovinge S, Olofsson PS.
Identification and Characterization of Human Activation-Induced $ChAT^{+}CD4^{+}$ T Cells.
BioRxiv, 2021, 441632. doi: 10.1101/2021.04.27.441632

II. **Gallina AL**, Rykaczewska U, Wirka RC, Caravaca AS, Shavva VS, Youness M, Karadimou G, Lengquist M, Razuvaev A, Paulsson-Berne G, Quertermous T, Gisterå A, Malin SG, Tarnawski L, Matic L[*], Olofsson PS[*].
AMPA-Type Glutamate Receptors Associated With Vascular Smooth Muscle Cell Subpopulations in Atherosclerosis and Vascular Injury.
Front. Cardiovasc. Med. 8:655869. doi: 10.3389/fcvm.2021.655869

III. Caravaca AS, **Gallina AL**, Tarnawski L, Tracey KJ, Pavlov VA, Levine YA, Olofsson PS.
An Effective Method for Acute Vagus Nerve Stimulation in Experimental Inflammation.
Front. Neurosci. 13:877. doi: 10.3389/fnins.2019.00877

IV. Caravaca AS, **Gallina AL**, Tarnawski T, Shaava V, Colas RA, Dalli J, Malin SG, Hult H, Arnardottir H, Olofsson PS.
Vagus nerve stimulation promotes resolution of inflammation by a mechanism that involves Alox15 and requires the α7nAChR subunit.
Manuscript

V. Botzanowski B, Donahue MJ, Silverå Ejneby M, **Gallina AL**, Ngom I, Missey F, Acerbo E, Byun D, Carron R, Cassarà AM, Neufeld E, Jirsa V, Olofsson PS, Głowacki ED, Williamson A.
Noninvasive Stimulation of Peripheral Nerves using Temporally-Interfering Electrical Fields
BioRxiv, 2021, 472557. doi: 10.1101/2021.12.14.472557

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

VI. Caravaca AS, Centa M, **Gallina AL**, Tarnawski L, Olofsson PS.
Neural reflex control of vascular inflammation.
Bioelectron Med. 6:3. doi: 10.1186/s42234-020-0038-7

VII. Christersdottir T, Pirault J, Gisterå A, Bergman O, **Gallina AL**, Baumgartner R, Lundberg AM, Eriksson P, Yan ZQ, Paulsson-Berne G, Hansson GK, Olofsson PS, Halle M.
Prevention of radiotherapy-induced arterial inflammation by interleukin-1 blockade.
Eur Heart J. 40(30):2495-2503. doi: 10.1093/eurheartj/ehz206

VIII. Brück E, Svensson-Raskh A, Larsson JW, Caravaca AS, **Gallina AL**, Eberhardson M, Sackey PV, Olofsson PS.
Plasma HMGB1 levels and physical performance in ICU survivors.
Acta Anaesthesiol Scand. 2021 Aug;65(7):921-927. doi: 10.1111/aas.13815

IX. Karadimou G, Gisterå A, **Gallina AL**, Caravaca AS, Centa M, Salagianni M, Andreakos E, Hansson GK, Malin S, Olofsson PS, Paulsson-Berne G.
Treatment with a Toll-like Receptor 7 ligand evokes protective immunity against atherosclerosis in hypercholesterolaemic mice.
J Intern Med. 2020 Sep;288(3):321-334. doi: 10.1111/joim.13085

X. Arnardottir H, Thul S, Pawelzik SC, Karadimou G, Artiach G, **Gallina AL**, Mysdotter V, Carracedo M, Tarnawski L, Caravaca AS, Baumgartner R, Ketelhuth DF, Olofsson PS, Paulsson-Berne G, Hansson GK, Bäck M.
The resolvin D1 receptor GPR32 transduces inflammation resolution and atheroprotection.
J Clin Invest. 2021 Dec 15;131(24):e142883. doi: 10.1172/JCI142883

XI. Brück E, Lasselin J, HICUS study group (Caravaca AS, **Gallina AL**, Bottai M, Eberhardson M, Sundman E), Andersson U, Sackey PV, Olofsson PS.
Prolonged elevation of plasma HMGB1 is associated with cognitive impairment in intensive care unit survivors.
Intensive Care Med. 2020 Apr;46(4):811-812. doi: 10.1007/s00134-020-05941-7

CONTENTS

LIST OF ABBREVIATIONS

α7nAChR	α7 nicotinic acetylcholine receptor
AA	Arachidonic acid
ACh	Acetylcholine
Alox15	Arachidonate 15-Lipoxygenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	Apolipoprotein E
ChAT	Choline acetyltransferase
CNS	Central nervous system
CRP	C-reactive protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
GF	Growth factor
Gln	Glutamine
GLS	Glutaminase
Glu	Glutamate
GLUL	Glutamate-ammonia ligase
GO	Gene ontology
GRIA	Glutamate receptor, ionotropic, AMPA type
hcSMC	Human carotid smooth muscle cell
HUVEC	Human umbilical vein endothelial cell
I_d	Inflammation decay
IFN	Interferon
IL	Interleukin
IL-1β	Interleukin-1 beta

KA	Kainate
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LN	Lymph nodes
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MΦ	Macrophage
NBQX	2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline disodium salt
NE	Norepinephrine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
ODE	Ordinary differential equation
OEPC	Organic electrolytic photo-capacitors
PBMC	Peripheral blood mononuclear cell
PBr	Pyridostigmine bromide
PE	Phenylephrine
PFA	Paraformaldehyde
PMN	Polymorphonuclear neutrophil
qPCR	Quantitative polymerase chain reaction
Ri	Resolution interval
scRNAseq	Single cell RNA sequencing
SPM	Specialized pro-resolving mediator
TENS	Transcutaneous electrical nerve stimulation
TI	Temporally-interfering
TINS	Temporal interference nerve stimulation
TLR	Toll-like receptor
TNF	Tumor necrosis factor

UHPLC-MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometry
VA-ECLS	Veno-Arterial Extra Corporeal Life Support
VN	Vagus nerve
VNS	Vagus nerve stimulation
VSMC	Vascular smooth muscle cell
VX	Vagotomy

1 INTRODUCTION

The immune system is a host defense system that protects from pathogens and orchestrates tissue healing after trauma. However, under certain conditions, the immune system can be more injurious than the pathogen invasion[12]. A highly controlled and organized immune response is of primary importance for health and survival. In fact, non-resolving inflammation leads to tissue damage and chronic disease, including autoinflammatory and cardiovascular diseases[6]. In recent years, neural reflexes have emerged as important regulators of inflammatory responses[1,13]. These discoveries have led to a significant increase in interest in understanding neural regulation of chronic inflammatory conditions and an ambition to develop novel bioelectronic medicine-based therapeutic solutions and diagnostic methods for inflammatory diseases based on the insights that activation of particular neural conduits regulate cytokine release and inflammation.

1.1 INFLAMMATION AND ITS RESOLUTION

In the first centuries of the current era, the concept of inflammation was known and characterized by five distinct qualities: *rubor* (redness), *calor* (heat), *tumor* (swelling), *dolor* (pain), and *functio laesa* (loss of function). These are the physiological results of immunological responses against harmful stimuli, such as injury, irritation, or infection caused by pathogens[14]. This is an active process that involves several components, including immune cells, the circulatory system and molecular mediators. Inflammation can be divided into acute and chronic[14].

Acute inflammation is a temporary response that involves first innate and then adaptative immunity. Inflammatory reactions are elicited shortly after the insult, and usually resolve in a few days. Inflammation initiation includes release of molecular mediators from the site of the insult. These molecules have multiple effects. First, they trigger blood vessel dilatation which facilitates extravasation of innate immune cells, in particular leukocytes, which can now reach the damaged tissue[15]. Polymorphonuclear neutrophils (PMN), the most abundant type of leukocytes in the blood, represent the main player at the early onset of inflammatory processes. PMNs infiltrate the damaged tissue in response to mediators and engulf and degrade their target. This phase is followed by a complex set of events that promote anti-microbial defense and healing, and includes the onset of resolution of inflammation[16]. Here, macrophages (MΦ) reach the site of the insult and clear out apoptotic PMNs, pathogens, and debris, in a process termed efferocytosis[17]. Changes of intracellular signaling pathways in MΦ regulate the progression and duration of the inflammatory response, resulting in distinct anti-inflammatory and pro-resolving outcomes[17,18]. This is reflected by the difference in mediators released by these two phenotypically different cells.

Anti-inflammatory responses aim to counter-balance and contain inflammation, while pro-resolving responses promote the cessation of inflammation via distinct active processes. Inflammation resolution consists in five processes: clearance of pathogens, apoptotic cells and cell debris, restoration of normal perfusion and vascular integrity, tissue healing, remission of fever, and relief from pain[17,19]. The fine balance between pro-inflammatory, anti-inflammatory, and pro-resolving forces is controlled by the complex interplay of several molecules with counter-acting actions. These mediators can be divided into different classes depending on their chemical structure, such as peptides, lipids and gasses[20].

Cytokines are small soluble proteins that are important mediators in orchestrating inflammation responses and host homeostasis[12]. Interleukins (IL), such as IL-6 and IL-1, interferons (IFN), and tumor necrosis factors (TNF) families are the principal pro-inflammatory cytokines that are released during injury or infection. However, this family of molecules is fundamental also for the resolution of inflammation. Resolution requires not only decreased PMN infiltration and increased efferocytosis of apoptotic cells and debris, but also counter-regulation of pro-inflammatory cytokines, often mediated by anti-inflammatory cytokines. For example, the anti-inflammatory cytokines IL-4 and IL-10 can inhibit the IL-1, TNF, and IFN signaling pathways[21,22]. The biosynthesis and release of pro-inflammatory cytokines can worsen inflammation. Cytokine-targeting drugs, such as inhibitors of TNF and IL-1, have been used for the treatment of rheumatoid arthritis and inflammatory bowel disease[23–25]. This approach has significantly improved the life quality of patients suffering from these conditions[26]. However, there are several drawbacks in using cytokine-inhibiting drugs, given by unwanted side effects, including significantly increased susceptibility to serious infections. Moreover, they are not effective for all patients, hence more selective treatment alternatives are needed.

Leukocytes at the site of the insult biosynthesize and release lipid mediators. Similar to cytokines, lipid mediators can be classified according to their pro-inflammatory or pro-resolving actions. Pro-inflammatory lipid mediators, such as eicosanoids, are important in the initiation of inflammation. Prostaglandins and leukotrienes, two of the subfamilies of the eicosanoids, regulate vascular permeability and migration of neutrophils to the site of injury, respectively[17–19,27,28]. To reach a complete resolution, lipid mediators class switch must occurr, from the initial inflammatory response to the following resolution of inflammation process[17,27]. Specialized pro-resolving mediators (SPM) are the product of several lipoxygenase (LOX) enzymes, which use essential fatty acids as substrates. These fatty acids include arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA)[27,28]. SPMs are divided into the following families: lipoxins, resolvins, maresins, and protectins. SPMs, limit neutrophilic infiltration, counter-regulate pro-inflammatory signals, promote recruitment of non-phlogistic monocytes, and stimulate efferocytosis, hence promoting inflammation resolution (**Figure 1**)[28–30].

Non-resolving inflammation can lead to chronic diseases[31,32]. Excessive and prolonged inflammatory stimuli result in the persistent migration of immune cells to the site of insult, amplifying the inflammatory response and aggravating tissue damage. Persistent inflammation is an important underlying cause of diseases such as atherosclerosis, diabetes, asthma, and rheumatoid arthritis[33]. Therapeutic approaches that implement strategies targeting pro-resolving processes, as well as anti-inflammatory ones, may be important in ameliorating these and similar chronic conditions.

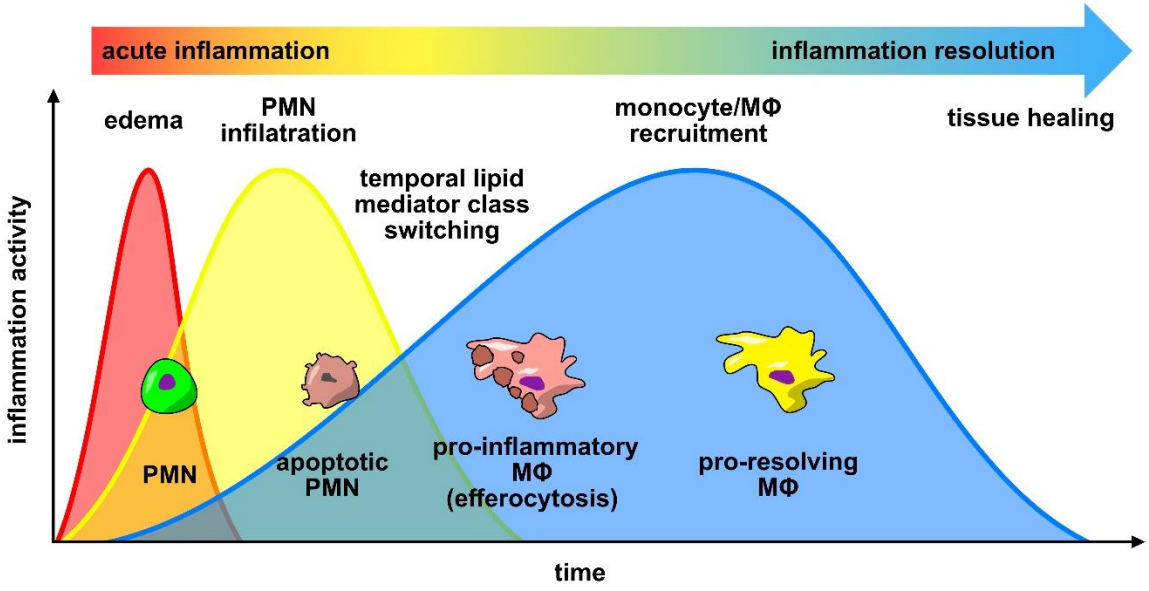

Figure 1: The onset and resolution of acute inflammation. The earliest stage of the inflammatory response is marked by tissue edema caused by increased blood flow and microvascular permeability and is mediated by the release of cytokines and proinflammatory lipid mediators. Polymorphonuclear neutrophils (PMN) infiltrate the site of injury in response to these mediators and initiate clearance of the pathogens. Subsequently, PMN become apoptotic and switch from releasing pro-inflammatory to pro-resolving mediators. These mediators activate macrophages (MΦ) to initiate efferocytosis. Moreover, PMN recruitment terminates and the MΦ switch to a pro-resolving phenotype promoting tissue repair.

1.2 NEURAL REGULATION OF INFLAMMATION

There are numerous mechanisms that help regulate inflammation and fight pathogens to protect the host and many of these processes involve neural reflex circuits[13,34,35]. Following infection or tissue damage, nerves can modulate immune cell activity including their release of

cytokines. Moreover, cytokines or lipid mediators released by immune cells activate neural signals and evoke local and central responses[36,37].

A neural reflex is defined as an involuntary precise response, elicited shortly after a stimulus. This response, which can be conscious or not, may be motor, secretory or vascular, and cannot be adapted according to circumstances[35]. The archetypical neural reflex has a sensory receptor which is activated by a defined stimulus in the environment, a sensory afferent arc carries the information, as action potentials, to a ganglion or the central nervous system (CNS). The signal is then processed and propagated via an efferent arc of the peripheral nervous system, ultimately impacting local physiology. For example, reflexes regulate the respiratory, gastrointestinal, and cardiovascular systems[35].

It has been shown that neural reflexes regulate immune responses[35,38,39]. This machinery is evolutionally ancient. Interactions between nervous and immune systems can be found in primitive simple organisms, such as *Caenorhabditis elegans* (*C. elegans*). *C. elegans* lacks mobile immune cells and an adaptive immune system, and the nervous system has the predominant role in protecting the host from pathoges[40]. Here, nervous fibers prevent excessive responses to infection and restore homeostasis, hence facilitating host survival after infection[40]. This underlies that neural control of immunity is not only primordial but fundamental since it has been preserved throughout the evolution of several animal kingdoms. In mammals, there is a variety of different interactions between nervous and immune systems. These two apparatuses work together to elicit several defense programs against pathogens and balanced inflammatory responses to insults[13]. Their interaction is key in regulation of inflammation from initiation to resolution, and neural reflexes can mediate complex bi-directional communication between the two systems[41].

Sensory signals are evoked by nociceptors, receptors that are activated by distress in the damaged tissue. They respond to numerous stimuli, including neurotransmitters, cytokines, and pro-inflammatory lipid mediators[42]. They are fundamental for local and systemic inflammatory responses. For example, nociceptor ablation corresponded to increased local inflammation in *Staphylococcus aureus* (*S. aureus*) infection in mice[43], while in a mouse model of sepsis had systemic inflammatory effects[44]. These observations place the sensory nervous system as an element of primary importance in the first phases of the inflammatory response. The motor responses elicited by the sensory signals may be various, given the intricate balance between sympathetic and parasympathetic systems. Sensory and sympathetic signals elicit a fast inflammatory response in the site of the insult, likely to prevent the spread of the infection, while the parasympathetic role is to modulate systemic responses to inflammation[45]. Sensory and sympathetic fibers can secrete various neurotransmitters, for example substance P or norepinephrine (NE). These molecules are known to actively increase the production of cytokines in immune cells; moreover substance P has chemoattractant proprieties for innate immunity cells[45].

Of note, it has been shown that not only neurons but also immune cells can biosynthesize and secrete neurotransmitters[46,47]. For example, peripheral blood mononuclear cells (PBMC) can biosynthesize catecholamines, including NE, epinephrine, and dopamine[47]. Granulocytes, T and B lymphocytes can produce and release dopamine[47]. Dendritic cells can secrete a high amount of the amino acid glutamate while MΦ, platelets, and mast cells are known to be serotonin producers[46]. As previously described, this type of chemical communication is key in the bi-directional crosstalk between the immune and nervous systems. In particular, ACh plays a key role as non-neuron-derived neurotransmitter in the inflammatory reflex[48].

1.3 THE INFLAMMATORY REFLEX

The best-characterized immune-regulating reflex is the "inflammatory reflex", in which the vagus nerve (VN) plays a central role regulating cytokine-release to maintain host homeostasis and prevent potentially damaging inflammation[11,49]. The VN is the 10th cranial nerve which arises from the medulla oblongata and with its afferent and efferent fibers, innervates the pharynx, larynx, trachea, lungs, heart, esophagus, stomach, liver, pancreas, small intestine, and proximal colon[50] (**Figure 2**). Additionally, it reaches several ganglia, e.g. the celiac ganglion, that project nerve fibers to more organs, one of which is the spleen, the main lymphoid organ in the body[51]. The VN represents the main component of the parasympathetic nervous system[52]. This nerve contains ∼20% of motor and ∼80% of sensory fibers, wrapped in a tunica of connective tissue[53]. Inflammatory stimuli activate the afferent branches of the nerve and elicit the neural reflex to suppress cytokine production and inhibit inflammation[3,11]. These responses can occur in a matter of seconds to minutes[54].

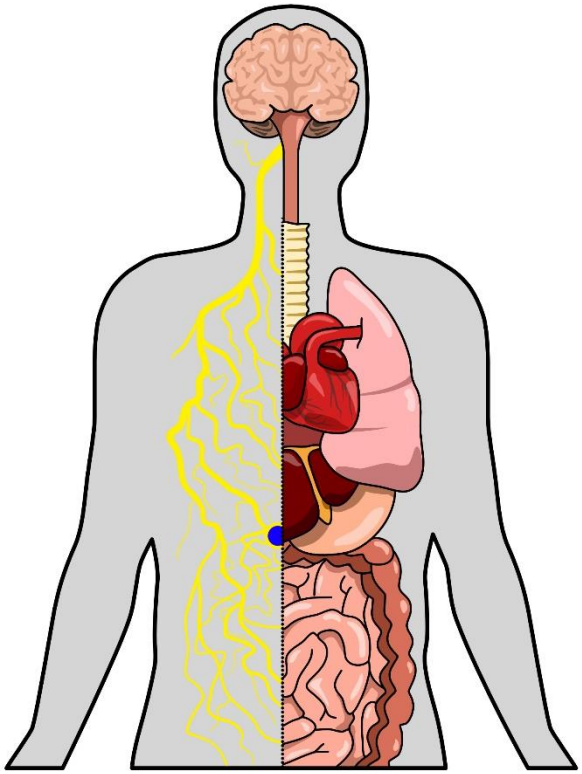

Figure 2: Schematic representation of the vagus nerve. Branches of the nerve are represented in yellow on the left, and the organs directly innervated by the vagus nerve are reported on the right. Organs represent brain, medulla oblongata, trachea, lungs, heart, stomach, liver, pancreas, small intestine and proximal colon. Celiac ganglion is represented in blue (left).

The sensory VN fibers can be activated by thermic, chemical, mechanical, and osmotic stimuli[55]. Several observations support the current model regarding the afferent branch of the inflammatory reflex. Sub-diaphragmatical vagotomy (VX) in rats leads to the absence of a fever response following an intraperitoneal injection of the cytokine interleukin-1 beta (IL-1β)[56]. Moreover, injecting IL-1β intravenously increases afferent splenic nerve activity in rats, but not if the VN hepatic branch is ablated[57]. These observations suggest that afferent signals of the VN, in response to IL-1β-injection, elicit a reflex-like motor response.

Regarding VN efferent fibers in the inflammatory reflex, it appears they impact inflammation in the periphery. VX in a murine endotoxemia model of inflammation resulted in increased serum TNF levels compared with sham-operated rats. Interestingly, if the VN was electrically stimulated (VNS), serum TNF levels were significantly decreased compared to both vagotomized and sham-operated rats[3]. In addition to inhibition of inflammation, VX caused longer resolution times and increased pro-inflammatory mediators when compared to sham in a mouse peritonitis model[58]. Additional experiments carried out *ex vivo* showed that electrical VNS on explanted tissue increased the biosynthesis of pro-resolving mediators and increased expression of netrin-1, a molecule involved in lowering prostaglandins production and neutrophil infiltration[58–60].

These and more observations led the inflammation neuroscience scientific community to establish a detailed anatomical and functional model of the afferent branch of the inflammatory

reflex (**Figure 3**). This model, broadly accepted with some minor exception[61], involves several components. Despite not being directly innervated by the VN, the spleen is the major lymphoid organ and has numerous key physiological roles in relation to the vascular and immunological systems. In murine models, it represents the primary source of TNF in systemic endotoxemia[62,63]. Efferent VN fibers carry signals to the left celiac ganglion, where the splenic nerve arises. However, this biology, and in particular details of the signal transmission in the ganglion, are not yet completely understood[64]. It has been observed that activation of the splenic nerve leads to the release of NE in the spleen, which activates adrenergic receptors expressed on lymphocytes[65], promoting release of acetylcholine (ACh) by choline acetyltransferase (ChAT)-expressing T cells[48,63,66,67]. ACh is the natural ligand of the α7 nicotinic acetylcholine receptor (α7nAChR), a pentameric ligand-gated ion-channel, expressed in the central and peripheral nervous systems. α7nAChR has been shown to suppress nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, via inhibition of intracellular Ca^{2+} release[68,69]. This directly impairs the production of pro-inflammatory cytokines[1,48]. Interestingly, this receptor is expressed on several non-neuronal cells including MΦ, monocytes, dendritic cells, endothelial cells, and T cells[70]. α7nAChR expressed on pro-inflammatory MΦ in the spleen represents the final component of the efferent branch of the inflammatory reflex, which regulates the biosynthesis and release of pro-inflammatory cytokines[71,72] (**Figure 3**).

In support of this model, genetically modified animals deficient in the α7nAChR exhibit an increased LPS-induced TNF production, compared to wild-type group mice[71]. Administration of the selective α7nAChR agonist GTS-21 attenuated release of pro-inflammatory cytokines in inflammation[73,74]. Similar results were achieved via VNS in experimental animals[3,71]. These observations indicate that the inflammatory reflex has anti-inflammatory effects. However, these studies fail to address whether efferent signals in the VN have a role in the resolution phase of inflammatory response.

As final note, mouse experiments have identified an additional role for one of the inflammatory reflex components. It has been shown that $ChAT^+$ $CD4^+$ T cells affect perfusion, hence extravasation. Vasodilatory proprieties of ACh are known to be mediated via muscarinic ACh receptors expressed on vascular endothelial cells. This activation increases nitric oxide (NO) release which directly acts on vascular smooth muscle cells (VSMC) inducing vessel relaxation[66]. $ChAT^+$ $CD4^+$ T cells can release ACh and promote the activity of NO-synthase, the enzyme which catalyzes the production of NO, in vascular endothelial cells[66]. Moreover, $ChAT^+$ $CD4^+$ T cells regulate not only systemic blood pressure but also microcirculation in inflammation and promote extravasation of T cells into virus-infected organs[67]. Moreover, it has been observed that surgical abrogation of VN signals resulted in a decrease of $ChAT^+$ $CD4^+$ T cell numbers, in a mouse sepsis model[75]. These observations indicate that the VN-mediated decrease of pro-inflammatory cytokines may be not the only outcome of efferent branch of the inflammatory reflex.

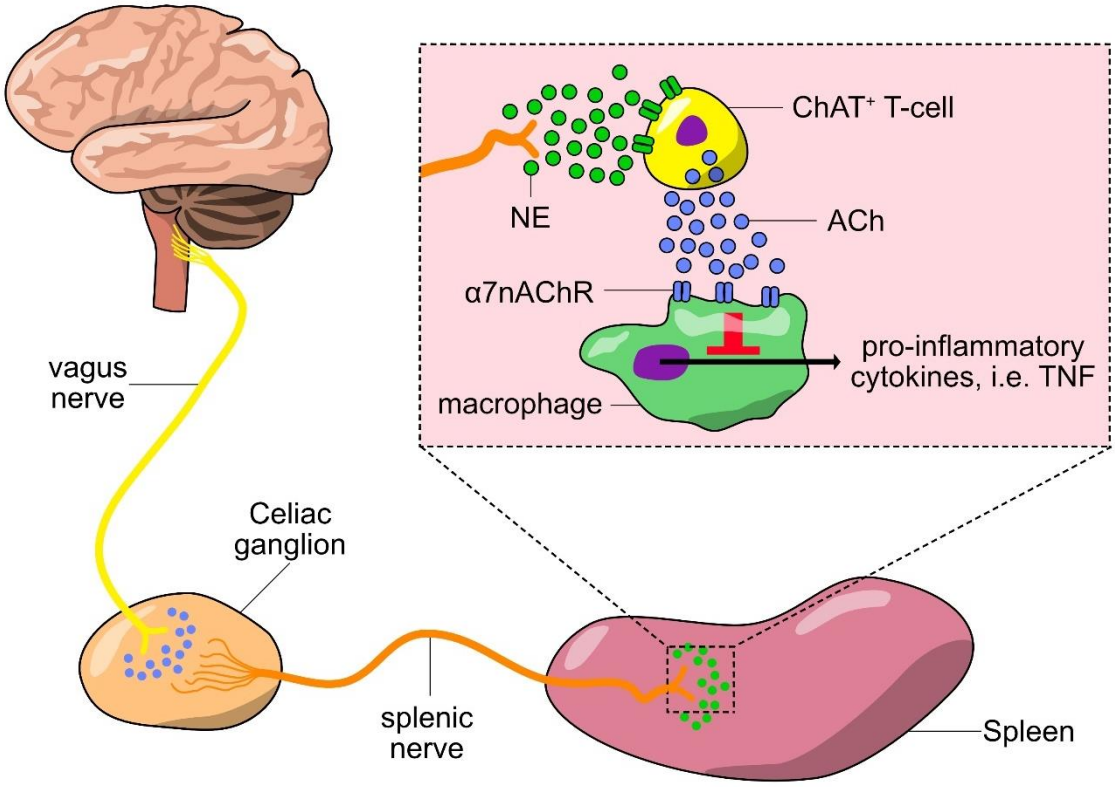

Figure 3: The efferent branch of the inflammatory reflex. Signals propagated in the cervical vagus nerve ultimately activate adrenergic neurons of the spleen. In the spleen, this signal is relayed by choline acetyltransferase $(ChAT)^+$ T cells that release acetylcholine (ACh) in response to norepinephrine (NE), activating α7 nicotinic ACh receptor (α7nAChR) on innate immune cells, suppressing release of TNF and other pro-inflammatory cytokines.

1.4 IMPLICATIONS OF NEURAL REGULATION ON CHRONIC INFLAMMATORY DISEASES

Firing activity and tissue innervation can change during the different phases of the acute inflammatory reaction[45]. Initially, local activity of sensory fibers increases and activate pro-inflammatory responses. The increase of sympathetic activity, which ensure availability of glucose and lipids, is instead systemic. However, sympathetic activity has inhibitory effects on inflammatory cells. Hence, while an active metabolism has to be kept in place during an inflammatory response, sympathetic activity has to decrease in the site of the insult[45]. It has been observed in arthritis patients, a local decrease of sympathetic innervation occurs, while sensory innervation increased[76]. This neural configuration, to be beneficial for the clearance of the antigens and apoptotic cells, must be temporary[45]. However, when this mechanism is defective, it may leads to non-resolving inflammation, which underlies chronic diseases[31,32].

Chronic diseases are characterized by persistent migration and activation of immune cells to the site of insult, amplifying the inflammatory response, aggravating tissue damage. This is associated with an impaired tissue healing and inflammation resolution[33].

It is unclear whether a sub-optimal nervous response is part of the cause or a consequence of non-resolving inflammation, and the characteristics of the neural response to non-resolving inflammation are hitherto not known. Partial paralysis, before the onset of chronic inflammatory disease, often corresponds to a lack of local inflammatory responses[77]. For example, sensory nervous fibers innervating the skin can promote the pathogenesis of psoriasis. Mice models lacking specific nociceptors failed to develop the expected psoriasis-like inflammatory reaction to a chemical insult[78]. Independently of whether the nervous system is responsible or not, to some degree, for the onsetting or severity of chronic disease, it represents also a potential key target element able to ameliorate these conditions.

1.5 CARDIOVASCULAR DISEASE AND VASCULAR SMOOTH MUSCLE CELLS IN ATHEROSCLEROSIS

Cardiovascular diseases (CVD) together with chronic inflammation causes disability, suffering, and death. Inflammation and failure of its resolution are key to the development and severity of several cardiovascular diseases. In fact, it has been shown that elevated levels of inflammatory markers, such as C-reactive protein (CRP), are associated with a higher risk of future cardiovascular events[79]. In 2017, CVD accounted for 45% of deaths in Europe[80]. Atherosclerosis is the underlying pathology behind the predominant clinical manifestations of CVD and consists of the formation of fibrofatty plaques in the intima layer of the artery wall (**Figure 4**). Atherosclerotic plaques cause myocardial infarction, ischemic stroke, as well as disabling peripheral artery disease[8]. There are well-known risk factors for the pathogenesis of atherosclerosis, including diabetes, cigarette smoking, hypertension, and high levels of low-density lipoprotein (LDL), a cholesterol-carrier protein present in the blood. Additionally, the immune system plays a key role in the onset and progression of this disease, and emerging risk factors include non-resolving inflammation[7]. Available treatments fail to sufficiently prevent atherosclerosis development and inflammation, to avoid death and disability from cardiovascular events[8].

There are reports connecting the pathophysiology of atherosclerosis with vascular smooth muscle cells (VSMC) phenotype[81]. VMSCs are constituents of normal arteries, providing structural and functional support to the tissue. These cells possess intrinsic plasticity and have the ability to be phenotypically modulated, playing a key role in atheroprogression[8,81,82]. In atherosclerotic plaques, VSMCs are key for plaque stability, since they are one of the main components in the protective fibrous cap[8]. Reduced stability of the plaque increases the risk of cardiovascular clinical events, such as heart attacks and stroke[7,8]. In recent lineage-tracing

studies, VSMCs appears to represent a larger proportion of cells than previously assumed. Since they are not terminally differentiated, they undergo phenotypical changes in response to local environmental stimuli, in both normal artery and atherosclerotic plaque[83–85] (**Figure 5**). VSMC proliferation seems to support vascular repair in atherosclerosis, however specific phenotypes of VSMC promote inflammation and plaque vulnerability[8,81]. Accordingly, understanding the regulation of VSMC phenotype may be relevant for improving risk assessment for adverse clinical events in atherosclerosis[81].

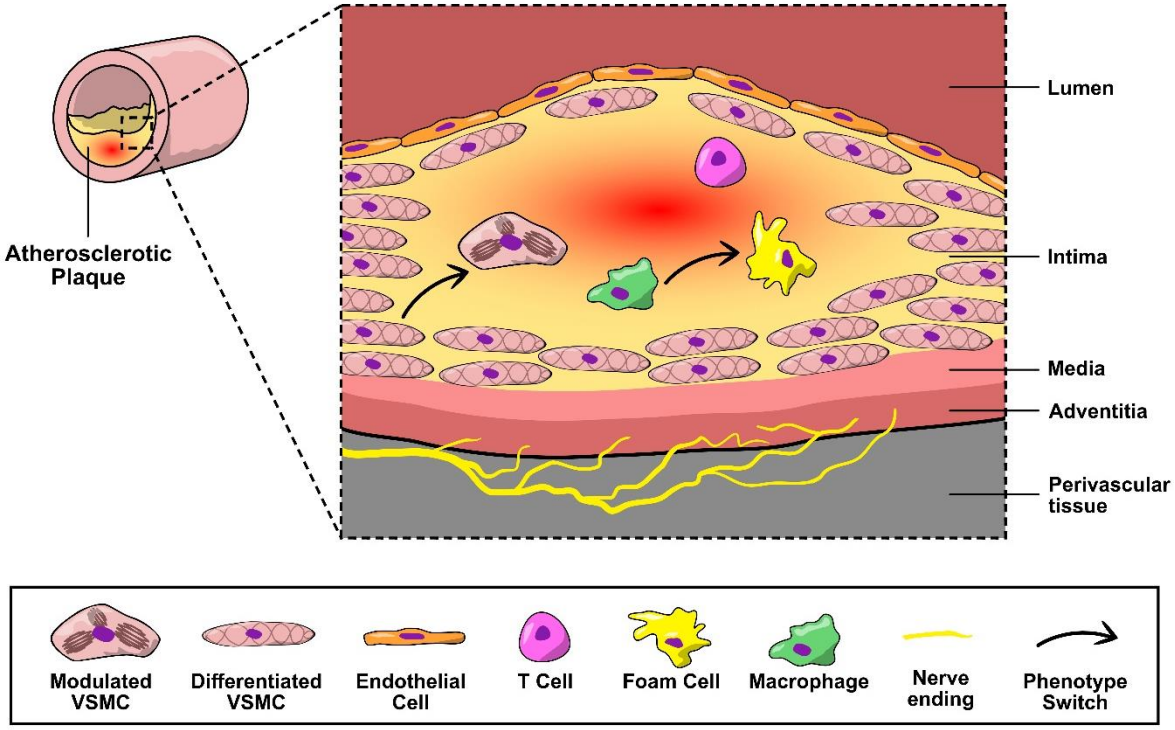

Figure 4: A schematic representation of the atherosclerotic plaque anatomy and formation in the arterial wall. Two types of cell phenotype change are represented: differentiated vascular smooth muscle cells (VSMC) differentiating into modulated SMCs and macrophages becoming lipid-rich foam cells.

Figure 5: Different characteristics and expression patterns of differentiated versus modulated vascular smooth muscle cells (VSMCs). Some of the stimuli that promote or inhibit the phenotypic switch are shown in the arrows. GFs = Growth factors; ECM = Extra cellular matrix.

1.6 THE AMINO ACID GLUTAMATE IN VASCULAR SMOOTH MUSCLE CELLS

Recent observations have highlighted a possible role for glutamate, a non-essential amino acid, in VSMCs in arterial remodeling. Glutamate, an important source of energy and a key signaling molecule, is present in plasma and synthesized from the precursor glutamine, which is the most abundant free amino acid[86,87]. It signals through receptors that are abundantly expressed into the CNS and several other tissues[88,89] (**Figure 6**). Glutamate ionotropic receptors are divided in several families, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-, N-methyl-D-aspartate (NMDA)-, kainate (KA)- and delta. They are found in the brain and also in the heart, skin, bone, and other organs, where their function has not been fully elucidated[89,90]. It has been observed in a mouse model of vascular remodeling, that deficiency of NMDA-receptors impaired VSMC proliferation[91]. While in a rat model of pulmonary hypertension, chemical block of glutamate receptors reduced vascular remodeling[91]. Other observations in humans, linked defects in glutamate metabolism and the pathophysiology of CVD. Glutamate-ammonia ligase (GLUL) is a key enzyme for glutamate turnover, a variant of this gene seemed to be responsible for coronary heart disease risk by affecting glutamate/glutamine metabolism[92]. Moreover, high glutamate blood levels were significantly associated with a higher CVD risk in paients[93].

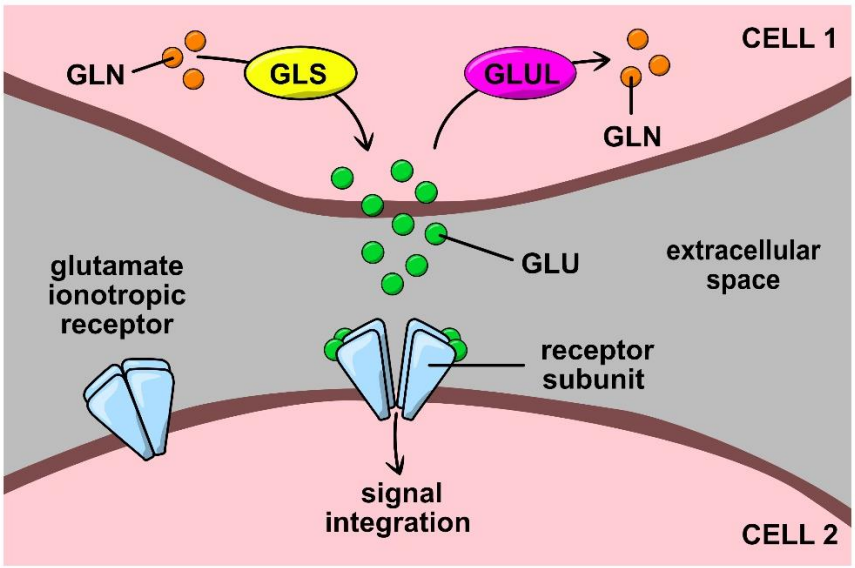

Figure 6: Schematic representation of cell-to-cell glutamatergic signalling via glutamate ionotropic receptor receptors (Gln = glutamine, Glu = glutamate, GLS = glutaminase, GLUL = glutamine synthetase).

1.7 INTERPLAY BETWEEN PERIPHERAL INNERVATION AND ATHEROSCLEROSIS

Cholinergic efferent vagal fibers innervate many organs, including the cardiovascular system. It is known that cholinergic signaling plays key roles in cardiovascular physiology, including regulation of heart rate[94] and blood pressure[10,95]. The ACh receptor α7nAChR is expressed in human atherosclerotic plaques and its activation attenuates atherosclerosis progression[96,97]. Moreover, it has been shown in an atherosclerosis-prone mouse model, treatment with inhibitors of acetylcholinesterase, the enzyme responsible for degrading ACh, resulted in anti-oxidative and anti-atherosclerotic effects[96]. Furthermore, arterial walls are innervated by adrenergic nerves, and efferent neural signals reach vascular SMCs, regulating vascular contraction[98]. It has been suggested that innervation of the vascular adventitia can be involved in neuro-immune crosstalk between layers of the vascular wall[9]. Pharmacological activation of α7nAChR in atherosclerosis-prone mice, treated with hypertension and angiotensin II, showed reduced blood pressure and suppression in the development of abdominal aortic aneurysm formation and atherosclerotic plaque[99]. In light of these observations, and the evidence that stimulation of the vagus nerve induces anti-inflammatory response and inhibition of pro-inflammatory cytokines, it is tempting to speculate that VNS might be utilized to reduce the inflammation of atherosclerosis and perhaps attenuate disease progression. It remains to be determined whether neurotransmitters and perhaps even neural reflexes are involved in the pathogenesis of atherosclerosis.

1.8 ELECTRICAL STIMULATION OF PERIPHERAL NERVES

Peripheral nerve stimulation has been performed since the 1970s as medical treatment[100]. The main clinical targets for nerve stimulation are the vagus, sacral and sciatic nerves[101]. VNS is already clinically approved for the treatment of certain types of epilepsy, and it is currently in clinical trials for the treatment of depression and obesity[102]. However, the previously reported findings indicate that VN activity regulate cytokine levels, hence inflammation. Electronic devices can activate the vagus nerve and attenuate inflammation, in both acute and chronic inflammatory diseases[63,65]. Currently, clinical trials based on VNS strategies are being designed and already carried out[103,104]. However, many of the neurophysiological, cellular and molecular mechanisms of this potentially important biology in inflammation and treatment of inflammatory disease, are currently unknown.

The activation of the inflammatory reflex and other inflammatory-resolvent pathways via electrical stimulation would likely provide a deeper knowledge of the molecular and physiological mechanisms of many inflammatory diseases. Additionally, it may represent a new therapeutic strategy for many of those chronic inflammatory diseases which are still lacking an effective treatment for their cure and prevention, e.g. atherosclerosis. Clinical trials utilizing vagus nerve stimulation are currently being carried out to provide and define therapeutic alternatives to drugs to decrease inflammation in a variety of conditions and diseases including rheumatoid arthritis and inflammatory bowel disease[105–109].

To improve significantly the study of inflammatory diseases *in vivo*, it is fundamental to design an effective method for consistent electrical VNS. It needs standardization in surgery, anesthesia, electrode placement, and stimulation parameters. In published animal studies, the protocol and methods used for VNS vary significantly. This may complicate interpretation and comparisons between reported observations[4,110,111]. Moreover, it has been shown that acute activation of the inflammatory reflex through VNS in an endotoxemia mouse model has significant anti-inflammatory effects which last for 48 hours after stimulation[112]. Hence, limitations in the application of VNS in animal models of chronic inflammatory diseases depend, *in primis*, on the unsuitability of the currently available devices for long-term implantation. A small, stable, wireless, and biocompatible device is needed to gain deeper insight into the role of VNS in conditions caused by non-resolving inflammation.

1.9 NEW METHODOLOGIES FOR CHRONIC NON-INVASIVE PERIPHERAL NERVE STIMULATION

Peripheral nerve stimulation is at the center of the entire bioelectronic medicine field. The full potential of this therapeutic approach is yet not adequately investigated or explored. This is not due to a lack of interest in the scientific community, it rather depends on the limits of the current technology and methodologies. Several problems need to be addressed before considering this relatively new tool at par with the more canonical therapeutical approaches, e.g. chemical drugs. Multiple fields are currently working on solving various aspects, including new bio-compatible materials for new nerve stimulation devices, new design for electrodes and

stimulators, new analytical approaches for nerve firing pattern recording and translation. However, at the current state, this technology has one crucial drawback which hasn't been yet successfully resolved: specific and reliable peripheral nerve stimulation is invasive.

Most of the acute and chronic VNS devices for therapeutic purposes need surgical implantation of electrodes, wires, and power sources that with time require multiple surgical battery changes. Moreover, additional problems need a solution in devices intended for animal research, due to the significantly inferior size of nerves and hosts e.g. for mice. Mouse models would be ideal in this context since the current availability of genetically modified and validated models, and the reduced costs and breeding times compared to larger animals. However, there is a lack of suitable chronic electrodes for mice and their designs are constantly mutating in attempts to achieve more reliable and reproducible outomes[113]. For these and other reasons, non-invasive stimulation solutions are of great interest.

Multiple approaches are currently being investigated. Focused ultrasound has been already utilized for peripheral nerve stimulation in mice, for example on the sciatic nerve[114]. Published findings regarding the use of this technology for VNS are currently lacking. Moreover, the precise mechanism by which ultrasound evokes action potentials is not yet known. Hypotheses include thermal modulation, mechanical effects, or intra-membrane cavitation[115–117]. Additionally, electromagnetic waves can be used to induce depolarization of the target cell membrane. This idea is at the foundation of organic electrolytic photo-capacitors (OEPC). OEPC are devices that create an electric current when exposed to a specific wave-length in the visible spectrum. This technology has been able to activate specific ion channels in an *in vitro* model using *Xenopus laevis* oocytes[118]. Adapted for use in mammals, these devices may replace the traditional, more invasive, wired electrodes. OEPC can be implanted on nerves and be activated by wavelengths above 600 nm since these frequencies can penetrate animal tissues, and evoke nerve activity wirelessly. This technology has already been used to stimulate the rat sciatic nerve *in vivo*[119]. One additional approach is transcutaneous electrical nerve stimulation (TENS), which could represent the solution to non-invasive stimulation of peripheral nerves. TENS-based devices are already been used in numerous clinical studies, and they are commercially available. However, this technology has limits due to the spatial resolution of this technology and to the current magnitudes which can safely be carried from the skin[120,121].

Temporal interference nerve stimulation (TINS) represents a relatively new promising approach for transcutaneous electrical nerve stimulation. TINS implements high-frequency temporally-interfering (TI) electric fields to more efficiently stimulate deeper targets compared to TENS[122]. The key aspect of this technology is that carrier waves frequencies are higher than 1 kHz, hence they can penetrate tissues but are unable to depolarize the neuronal membrane. However, nerve activity can be induced in a focal point, where two phase-shifted frequencies interfere, reaching an offset frequency that is low enough to evoke action potentials[123] (**Figure 7**). In this thesis, for the first time, TINS was successfully used as a stimulation methodology for peripheral nerve *in vivo*.

Taking together these new approaches may help to provide an effective method for chronic non-invasive peripheral nerve stimulation, hence helping to target specific key elements in pathology and treatment of inflammatory diseases.

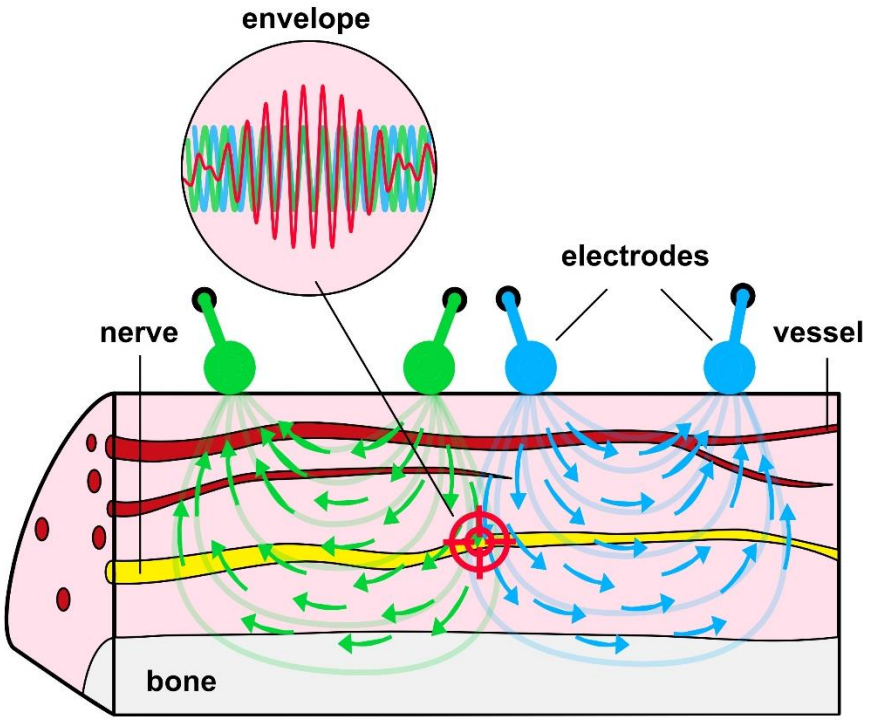

Figure 7: Concept of temporal interference nerve stimulation, in which two pairs of electrodes generate two high-frequency currents at a small frequency difference, resulting in oscillating electric fields. This, leads to low-frequency envelopes in target confined regions, where nerves lay.

2 RESEARCH AIMS

This thesis aims to investigate mechanistic aspects of neural regulation of inflammation including the ambition to improve on techniques for peripheral nerve stimulation in experimental models of chronic inflammatory diseases.

The specific aims for the projects in this thesis were:

I. Investigate whether human T cells participate in cholinergic regulation of vascular function (Project I).

II. Study nervous system associated factors in human atherosclerosis (Project II).

III. Describe an effective and practical method that facilitates cross-laboratory reproducibility in a mouse model of systemic acute inflammation (Project III).

IV. Investigate whether electrical activation of the cervical vagus nerve promotes resolution of inflammation in experimental peritonitis (Project IV).

V. Develop a non-invasive technology with improved specificity for peripheral nerve stimluation in experimental mice (Project V).

3 MATERIALS AND METHODS

3.1 *IN VITRO* EXPERIMENTS

3.1.1 Isolation and activation of human primary T cells

Whole blood from healthy individuals was purchased from Karolinska University Laboratory (Department of Immunology and Transfusion Medicine). PBMCs were isolated and cryopreserved in liquid nitrogen. Depending on the experiment, $CD3^+$ or Naive $CD4^+$ T cells were isolated using magnetic isolation kits. T cells were activated using anti-CD3 and anti-CD28 antibodies and maintained in culture for 0-14 days.

3.1.2 Stimulation of human carotid smooth muscle cells (hcSMCs)

2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline disodium salt (NBQX) was used as selective AMPA/KA ionotropic glutamate receptor antagonist[124]. Primary human carotid smooth muscle cells (hcSMCs) of passage 5–7 were serum-starved for 24 h, then the media was replaced with NBQX-supplemented media with a concentration of 25 μM, for the next 24 h. Supernatant and cells were collected for downstream analyses. Phenotypic characterization of these cells, via expression analysis of contractile markers, has been performed previously[125].

3.1.3 Isolation of mouse macrophages

Mouse cells derived from peritoneal lavage were collected after 12 hours since zymosan intraperitoneal injection. Cells were then plated on fibronectin-coated glass coverslips in well plates for cell culture. After macrophages attachment (~ 4 hours) coverslips were washed with PBS and then fixed with 4% PFA for further immunofluorescent analysis.

3.2 *EX VIVO* EXPERIMENTS

3.2.1 Myograph

Activated healthy human T cells were isolated into PBS in the presence of pyridostigmine bromide (PBr) and incubated for 15 minutes to make cell-free conditioned supernatant. Descending mouse thoracic aortas were harvested and arterial rings were mounted on a myograph system. After an equilibrating period, vessels were pre-constricted with phenylephrine (PE) and endothelial function was assessed by ACh dose-response curve measurements, or vehicle, with and without pre-treatment of the vessel with atropine. All myograph measurements were performed blinded.

3.3 *IN VIVO* EXPERIMENTS

3.3.1 Lipopolysaccharide (LPS)-induced endotoxemia

A batch-dependent dose of the toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) was administered intraperitoneally, after 1 hour of recovery from VNS or sham surgery. Mice were euthanized by carbon dioxide asphyxiation 90 minutes after injection. Blood was collected for later analysis of serum TNF and other mediators of interest.

3.3.2 Zymosan-induced peritonitis

0.1 mg of the toll-like receptor 2 (TLR2) ligand zymosan was administered intraperitoneally, after 1 hour of recovery from VNS or sham surgery. Mice were euthanized by carbon dioxide asphyxiation at 4, 12, 24, and 48 hours after zymosan challenge. The peritoneal exudate was collected by injecting PBS intraperitoneally, shaking the abdomen, and recovering the exudate. Cells were counted and fractions of each population were determined by flow cytometry analysis.

3.3.3 Acute vagus nerve stimulation (VNS)

Mice were anesthetized with isoflurane and temperature of the homeothermic pad was set at 37°C. The neck area was shaved and the left cervical branch of the vagus nerve was exposed and suspended on a custom-built platinum-iridium hook electrode. Constant current stimulation was applied to the nerve at 1 mA. A charge-balanced biphasic square waveform for stimulation was used, composed of 250 μs biphasic pulse and 50 μs interphase delay, at 10 Hz for 5 min. After stimulation, salivary glands and tissues were restored to position and the skin was sutured. Mice in the sham group were subjected to surgery, but not to electrical stimulation.

3.3.4 Vagotomy

Similar to the VNS surgical procedure, the left cervical branch of the vagus nerve was isolated and suspended. A segment of the nerve (~2–3 mm) was then removed to permanently disrupt nerve signals. After resection, salivary glands and tissues were restored to position and the skin was sutured.

3.3.5 Non-invasive sciatic nerve stimulation

Mice were anesthetized with isoflurane, and the temperature was monitored at 37°C and maintained through a homeothermic pad. Fur was removed on the leg to assure full contact of the 4-pin gold-plated header used for stimulation of the sciatic nerve. Stimulation parameters (waveforms and current amplitude) were provided by a two-part system. The two electrodes, both composed by the stimulators and their grounds, were connected to individual current generators, in turn, connected to independent function generators.

3.4 ANALYTICAL TECHNIQUES

3.4.1 Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatant from cell culture and serum from blood were collected in different defined conditions, depending on the experiment. They were diluted when needed and analyzed for cytokines and chemokines concentration by ELISA (mono- or multi-plex immunoassays), performed according to manufacturer instructions.

3.4.2 Quantitative polymerase chain reaction (qPCR)

RNA was isolated using an RNeasy kit with DNase treatment. cDNA was generated using an RNA-to-cDNA Kit. qPCR reactions were performed by mixing pre-diluted cDNA with the 20x TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix. The qPCR was run using a real-time PCR system. All samples were analyzed in duplicate. Results were normalized to PPIA, as a house-keeping gene, unless differently stated.

3.4.3 Immunofluorescence

3.4.3.1 Tissue

Human carotid plaques were fixed in 4% PFA and embedded in paraffin. Slides were deparaffinized and rehydrated in graded ethanol. Slides were subjected to high-pressure boiling in pH 6.0 buffer for antigen retrieval. Thereafter, slides were treated with blocking media before incubation with primary antibodies (~16 hours). Secondary antibodies were biotin-conjugated, labeled with fluorophore-conjugated streptavidin, used for the detection. Nuclei were stained with DAPI and slides were mounted with mounting media. Images were acquired with a confocal microscope.

3.4.3.2 Cultured cells

hcSMCs cultured on fibronectin-coated glass coverslips were washed with PBS and fixed with 4% PFA. Cells were treated with blocking media before incubation with primary antibodies (~16 hours). Secondary antibodies were biotin-conjugated, labeled with fluorophore-conjugated streptavidin, used for the detection. Nuclei were stained with DAPI and coverslips were mounted with mounting media. Images were acquired with a confocal microscope.

3.4.4 Immunohistochemistry

PFA-fixed tissue sections were deparaffinized and rehydrated in graded ethanol. For antigen retrieval, slides were subjected to high-pressure boiling in pH 6.0 buffer. The slides were stained with Hematoxylin QS, dehydrated and mounted. Images were acquired using an automated slide scanner system.

3.4.5 Liquid chromatography-tandem mass spectrometry

In human studies, T cells were isolated into PBS in the presence of PBr and incubated for 30 minutes. The conditioned supernatant was snap-frozen and cryopreserved until analysis. Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was used to measure concentrations of ACh. The calibration standard curves were prepared in PBS. All chromatography measurements were blinded.

In mouse studies, peritoneal exudates were collected and snap-frozen and cryopreserved until analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to measure concentrations of lipid mediators. Each lipid mediator was identified using the following criteria: at least 8 data points, signal-to-noise ratio higher than 5, and presence of a peak matching with the respective standard. The calibration standard curves were prepared in synthetic compound mixtures.

3.4.6 *In silico* analysis

3.4.6.1 Gene set enrichment analyses

3.4.6.1.1 Biobank of Karolinska endarterectomies (BiKE) cohort

Gene set enrichment analyses on Gene Ontology (GO) terms were performed with GeneMania and GOrilla software. Overlapping of GO categories was assessed using Revigo software.

3.4.6.1.2 RNAseq data from hcSMCs

Gene set enrichment analyses on GO terms were performed by using R/Bioconductor package topGO[126]. Enrichment was assessed using the Kolmogorov-Smirnov testing method. Additional data about the genes were extracted with GeneCards and PubMed. To visualize changes in the signaling pathway genes R/Bioconductor packages KEGGREST and Pathview were used[127,128].

3.4.6.2 Resolution of inflammation indices analyses

3.4.6.2.1 Halving of neutrophil peak interval

The previously described method[129] requires the time point of maximum neutrophil count and the time point which corresponds to a 50% decrease from the neutrophil count peak. The resolution index is defined as the time interval between the two values.

3.4.6.2.2 Exponential decay model

The neutrophil counts over time was analyzed in multiple publicly available datasets and we observed that there was exponential decay of neutrophil counts over time after

the peak. Accordingly, an exponential decay model was used for analysis of the reduction in neutrophil numbers over time, e.g. the inflammation decay, in subsequent experiments. The course of inflammation, as represented by neutrophil counts over time, was visualized using an ordinary differential equations (ODE) model.

3.5 STATISTICAL ANALYSIS

Differences between two experimental groups were assessed using a one- or two-tailed Student's t-test as appropriate (Study I, II, III, IV). Differences between multiple experimental groups Two-tailed Student's t-test corrected for multiple comparisons via the Bonferroni-Dunn method, one-(Study II, III) or two-way (Study IV) ANOVA followed by Fisher's Least Significant Difference (Study IV) or by Dunn's or Dunnett's multiple comparisons post-hoc tests (Study I) and Kruskal–Wallis followed by Tukey's multiple comparisons post-hoc tests (Study I) as appropriate. Spearman (Study I) or Pearson (Study II) correlations were used to determining the association between two continuous variables. Grubbs' outlier test was used as applicable (Study III, IV). Values are shown as mean ± SEM unless otherwise stated (Study I, II, III, IV). $p \leq 0.05$ was considered significant.

3.6 ETHICAL CONSIDERATIONS

3.6.1 Human studies

3.6.1.1 The biobank of Karolinska endarterectomies (BiKE) Cohort

Patients undergoing surgery for carotid stenosis at the Department of Vascular Surgery (Karolinska University Hospital, Sweden), were enrolled in the study, and clinical data were recorded on admission. Patients without qualifying symptoms within 6 months prior to surgery were categorized as asymptomatic. The BiKE study cohort demographics, details of sample collection, processing, and analyses were previously described[130]. The studies are approved by the Ethical Committee of Stockholm and follow the guidelines of the Declaration of Helsinki.

3.6.1.2 Veno-arterial extra corporeal life support (VA-ECLS) study

The VA-ECLS study was approved by the Spectrum Health Review Board. As participation criteria, all patients or their legally authorized tutors gave informed consent. All samples and data were anonymized before analysis.

3.6.1.3 Coronary artery plaques

Coronary arteries were dissected from explanted hearts of transplant recipients who provided their written consent prior to the procedure and their inclusion in this cohort.

Tissues were obtained from the Human Biorepository Tissue Research Bank under the Department of Cardiothoracic Surgery, with approval from the Stanford University Institutional Review Board. The basic clinical characteristics of the patients included (n = 4) in the study were previously described[131].

3.6.2 Animal studies

3.6.2.1 Rat carotid artery injury and healing response model

Animal studies were performed with the approval of the Ethical Board of North Stockholm and conform to the guidelines from the European Parliament on the protection of animals used for scientific purposes.

3.6.2.2 Nerves stimulation and ablation in mouse

This study and all experimental protocols were approved by the Stockholm Regional Board for Animal Ethics (Stockholm, Sweden).

3.6.2.3 Atherosclerosis prone mouse model

The animal study protocol was approved by the Administrative Panel on Laboratory AnimalCare at Stanford University.

3.6.2.4 Mouse arterial relaxation assay

Animal experiments were performed under protocols approved by the Animal Ethics Review Board of Stockholm, Sweden, in accordance with guidelines from the Swedish National Board of Laboratory Animals.

4 RESULTS & DISCUSSION

4.1 PROJECT I

4.1.1 Activated primary human T cells express *ChAT* and release ACh

Aimed to characterize $ChAT^+$ T cells in humans, we isolated primary T cells from the blood from healthy individuals. *In vitro*, cells were activated using anti-CD3 and anti-CD28 antibodies and mRNA was extracted at 0, 24, 48, 72, 96, 120 hours. Timepoints of 72 and 96 hours showed a significant increase in levels of *ChAT* mRNA and ACh the supernatant, respectively, when compared to the 0 hour timepoint (**Figure 8A, 8B**). Flow cytometry analysis showed that the majority of the activated T cells at the 96 hour timepoint were characterized by a large size and stained positive for the activation marker $CD25^{132}$ (**Figure 8C**). Interestingly, only the sub-population of larger activated T cells had detectable *ChAT* mRNA and significantly higher ACh levels at the 96 hour timepoint, compared to the sub-population of smaller cells (**Figure 8D, 8E**).

Interestingly, these findings are in line with previous observations in animal studies, where *ChAT* mRNA levels or $ChAT^+$ cell number were increased after T cell activation *in vitro* and infection *in vivo*, respectively[48,67]. Moreover, ACh biosynthesis plays a role in inflammation and vascular physiology[66,133]. Accordingly, we next investigated whether T cell-conditioned supernatants promoted vascular relaxation.

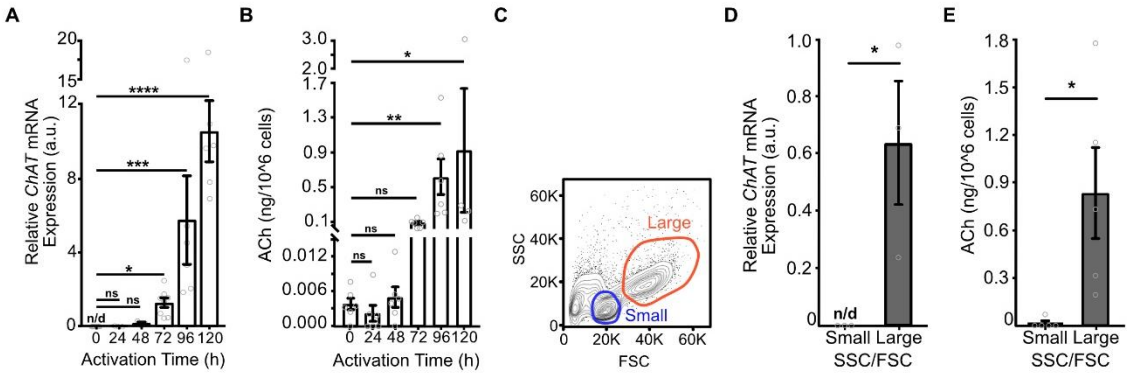

Figure 8: (**A**) qPCR quantification of *ChAT* mRNA extracted from activated primary human (n = 3-8) T cell at indicated time points. Values were normalized to *PPIA* and *B2M*, graphed relative to the 72 hours timepoint (Kruskal–Wallis test, post hoc Dunn's multiple comparisons test). (**B**) Mass spectrometry quantification of ACh released in supernatant from activated human (n = 6) lymphocytes. (Kruskal–Wallis test, post hoc Dunn's multiple comparisons test). (**C**) Flow cytometry gating for sorting of activated primary human lymphocytes sub-populations at 96 hours timepoint. (**D**) qPCR quantification of *ChAT* mRNA in primary human (n = 3) lymphocytes from (C). Values were normalized to PPIA, and expressed relative to large cells (Two-tailed, unpaired Student´s t-test). (**E**) Mass spectrometry quantification of ACh released in supernatant from large and small human (n = 3) activated T cells isolated in (C). (Two-tailed, unpaired Student´s t-test). SSC/FSC = side scatter/forward scatter. n/d – not detected, ns – not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.1.2 T cell-derived ACh induces vasorelaxation

ACh promotes vasorelaxation via activation of the muscarinic ACh receptor expressed on the endothelial cells of the vascular walls, and activation of endothelial nitric oxide synthase (NOS). This, results in NO release and vascular SMCs relaxation. In mice, T cells producing ACh regulate blood pressure and perfusion[66,67]. A mouse aortic vascular tension assay was used to investigate the effect of human T cell-conditioned supernatants on vascular relaxation. Exposure of *ex vivo* pre-constricted aortic rings to conditioned supernatants resulted in significant vasorelaxation, as compared to control. No effect was observed in aortic rings pre-treated with the muscarinic receptor blocker atropine (**Figure 9A**). *In vitro*, T cell-conditioned supernatant increased release of NO related species in human umbilical vein endothelial cells (HUVECs), as compared to vehicle exposure (**Figure 9B**). Activated T cells were divided into $CD3^+$ and $CD4^+$, the conditioned media from these two sub-populations significantly increased the production of NO-related species in human endothelial EA.hy926 cells, compared to control. This effect was abolished by atropine (**Figure 9C**). The T cell density and resulting ACh concentration in these experiments are in a physiological range[134].

These observations indicate that the release of human T cell-derived ACh to the extracellular space is sufficient to promote arterial relaxation through muscarinic ACh receptor activation.

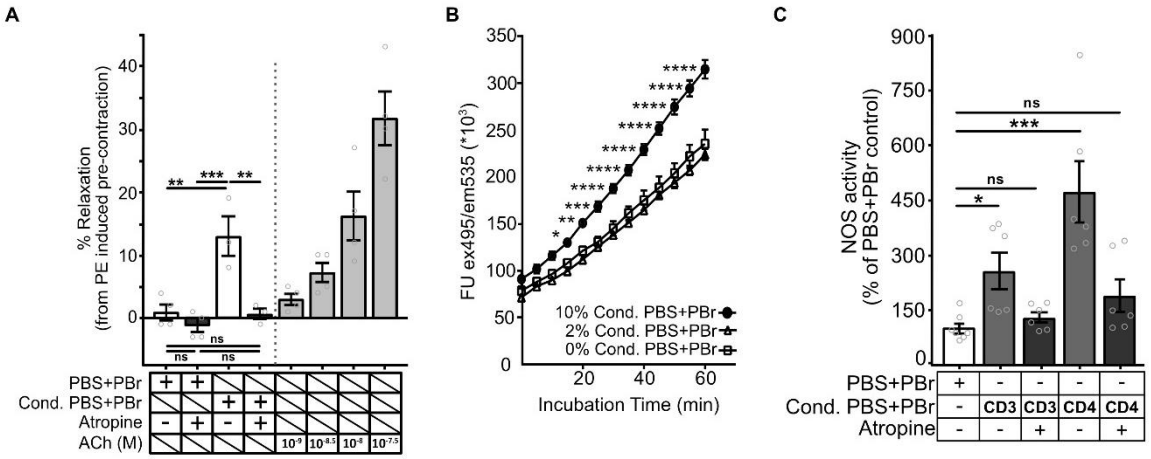

Figure 9: (**A**) Vascular relaxation of vessels after exposure to activated human T cell-conditioned PBS. Values are compared to maximal phenylephrine (PE)-induced pre-contraction. (left) Pre-contracted mouse aortas were exposed to vehicle (n = 4) or conditioned PBS (n = 3), with or without atropine pre-treatment (One-way ANOVA, Tukey's multiple comparisons test). (right) Pre-contracted mouse aortas were exposed to ACh at indicated concentrations. (**B**) 4-amino-5-methylamino-2',7'-difluorofluorescein fluorescence intensity assay measurements of NO related species released from HUVEC cells following exposure to vehicle (n = 3) or activated T cells conditioned supernatants (n = 6) at 10% or 2% (Two-way ANOVA, Dunnett's post hoc test). (**C**) Colorimetric Griess assay measuring of NOS activity in EA.hy926 endothelial cells following exposure to activated human $CD3^+$ (n = 2) or $CD4^+$ (n = 3) T cell-conditioned supernatant, with and without atropine. (Kruskal–Wallis, Dunn's multiple comparisons post-hoc test). ns – not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.1.3 Primary $CD4^+$ T cells express ChAT and their frequency correlated with survival in critically ill patients

We next investigated $ChAT^+$ $CD4^+$ T cells in human clinical patients. Single-cell RNA sequencing (scRNAseq) publicly available datasets of immune cells profiling in healthy individuals (n = 11) detected no ChAT expression in single $CD4^+$ T cells (**Figure 10A**). This is consistent with our previous observation regarding blood-derived human T cells from healthy individuals, where *ChAT* mRNA was not detected in non-activated cells, and its expression was induced after prolonged cell activation (Figure 8A). We then investigated *ChAT* in a cohort of critically ill patients with circulatory failure in need of Veno-Arterial Extra Corporeal Life Support (VA-ECLS) (n = 33). These patients are characterized by elevated

plasma levels of pro-inflammatory cytokines indicating ongoing inflammation. scRNAseq revealed that all patients had detectable $ChAT^+$ $CD4^+$ T cells in the blood (**Figure 10A**).

We investigated whether the relative frequency of $ChAT^+$ $CD4^+$ T cells in blood correlated to survival in this cohort. In a Cox proportional hazard rate model, patients in the 25-percentile of $ChAT^+$ $CD4^+$ cells frequency had a 5.9-fold higher hazard rate for 30-day mortality compared to patients in the 75-percentile. The other co-variates at their median, such as sex, inotrope score, and lactate[132]. The corresponding survival probability for $ChAT^+$ $CD4^+$ cell relative frequency is represented in **Figure 10B**.

This observation shows a correlation between mortality and relative abundance of $ChAT^+$ $CD4^+$ T cell, placing low $ChAT^+$ $CD4^+$ T cell frequency in blood as a risk factor in this cohort. As previously reported in mice[67], $ChAT^+$ $CD4^+$ T cells may promote vasodilatation and extravasation. Hence, high number of $ChAT^+$ $CD4^+$ T cells could improve pathogen clearance and organ perfusion, contributing to the recovery in critically ill patients. However, considering the limited sample size of the cohort, it is fundamental to further study mechanisms and effects of $ChAT^+$ $CD4^+$ T cell-mediated vasorelaxation in human disease.

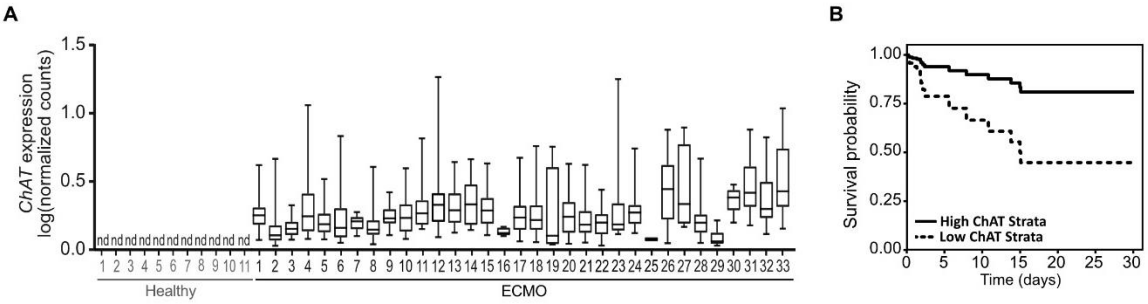

Figure 10: (**A**) scRNAseq data on *ChAT* expression in peripheral blood $CD4^+$ T cells from critically ill patients (n = 33) and healthy control individuals (n = 11) from publicly available datasets. The boxes indicate the interquartile range, the central bar indicates the median, and the whiskers show the 5th to 95th percentile range. (**B**) Cox proportional hazard rate model for the survival probability for patients (n = 32) with low $ChAT^+CD4^+$ T cell frequency (Low ChAT Strata, 25th percentile) and high $ChAT^+CD4^+$ T cell frequency (High ChAT Strata, 75th percentile). nd: not detected.

4.2 PROJECT II

4.2.1 Nervous system-associated transcripts are expressed in atherosclerotic plaques and segregate glutamatergic signaling components

Despite atherosclerotic lesions being not directly innervated, mouse experiments show that peripheral nerve activity regulates atherosclerosis inflammation[135]. Additionally, it has been reported that the key component of the inflammatory reflex α7nAChR is expressed in human atherosclerotic plaques, and receptor activation attenuates atherosclerosis progression[96,97]. The mechanism and potential relevance of neural regulation of inflammation in atherosclerotic lesions remain hitherto unclear. Here, we investigated the expression of nervous system-associated transcripts in human atherosclerosis. We compiled a list of distinctive components of the central and peripheral nervous systems, each classified by function or cell type association (**Figure 11A**). We analyzed microarray data of atherosclerotic plaques from the BiKE cohort, stratified in asymptomatic and symptomatic patients. We identified transcripts with significantly different mRNA levels, between which the most prominent were associated with glutamine signaling. In particular, *GLUL* was the most upregulated gene in symptomatic patients, while glutamate receptor, ionotropic, AMPA type subunits 1 and 2 (*GRIA1* and *GRIA2*) were significantly downregulated in this group, as compared with the asymptomatic patient group (**Figure 11B**).

This analysis revealed significant differences in transcript levels between atherosclerosis patients classified as symptomatic and asymptomatic in the BiKE cohort. It has been reported that neurotransmitter receptors are involved in arterial remodeling and pathophysiology of atherosclerosis[97,136]. In particular the glutamatergic axis components may represent a starting point for further exploration and discovery of key nervous-system-related signaling in atherosclerosis.

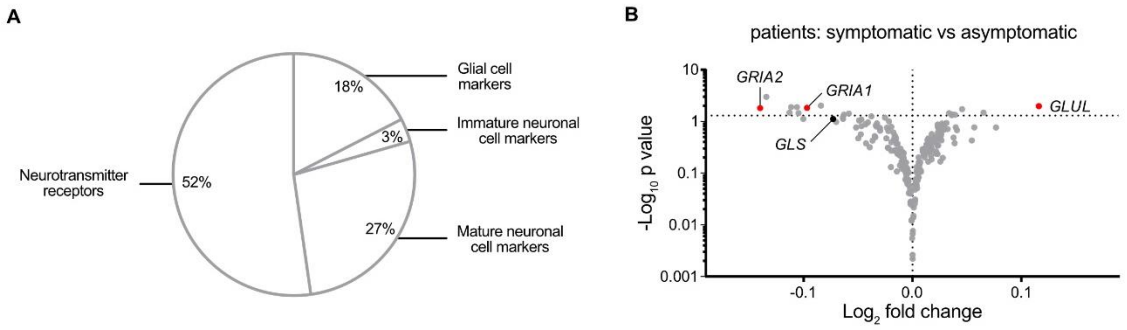

Figure 11: (**A**) Classification of the 217 nervous system-associated genes investigated by expression analysis in the microarray data from the Biobank of Karolinska Endarterectomies (BiKE). (**B**) Visualization of differentially expressed genes between symptomatic (n = 87) and asymptomatic (n = 40) patient groups in the BiKE cohort. Differently expressed Glutamatergic signalling-related genes are shown in red when significant, otherwise in black (unpaired Student's t-test, Bonferroni-Dunn multiple comparisons test). *GRIA1*, Glutamate Ionotropic Receptor AMPA-type Subunit 1; *GRIA2*, Glutamate Ionotropic Receptor AMPA-type Subunit 2; *GLS*, Glutaminase; *GLUL*, Glutamate-ammonia ligase.

4.2.2 Low mRNA AMPA-Type receptor expression in atherosclerotic plaques is associated with severe clinical events

Next, we investigated whether *GRIA1* and *GRIA2* mRNA levels were associated with the severity of adverse clinical events in the BiKE cohort. Based on the severity of clinical presentation at the time of surgery, patients were stratified into three groups: transient ischemic attack (TIA) and minor stroke (MS) were considered severe clinical events; amaurosis fugax (AF) was considered a minor adverse symptom; and asymptomatic patients. Levels of *GRIA1*, *GRIA2*, *GLS*, and *GLUL* were compared between groups. *GRIA1*, *GRIA2*, and *GLS* transcripts were significantly lower, while *GLUL* was significantly higher, in the group with the most severe symptoms (**Figure 12A**). To investigate the presence of mediators associated with glutamate signaling and metabolism in human atherosclerotic plaques, we queried BiKE cohort data for expression of ionotropic glutamate receptors of NMDA-, AMPA-, KA- and delta-type subunits and enzymes associated with glutamate biosynthesis. *GRIA1*, *GRIA2*, *GLS*, and *GLUL* transcripts were detected in biopsies from both non-atherosclerotic reference arteries and atherosclerotic lesions (**Figure 12B**).

These observations indicate that the machinery for glutamate turnover and signaling is present in human atherosclerotic lesions, and they may represent the ground for further experiments to investigate whether drugs promoting glutamatergic signaling are beneficial in atherosclerosis.

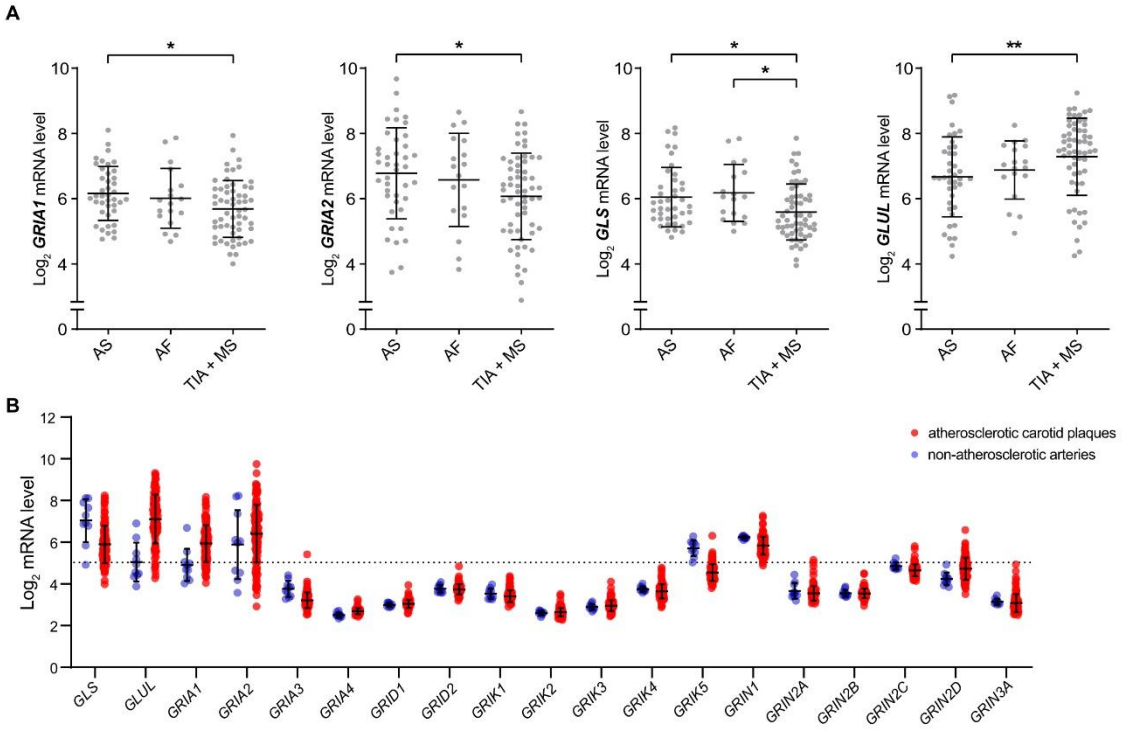

Figure 12: (**A**) From left to right, comparison of *GRIA1*, *GRIA2*, *GLS*, and *GLUL* mRNA levels between asymptomatic (AS) (n = 40), amaurosis fugax (AF) (n = 18), and transient ischemic attack + minor stroke (TIA + MS) (n = 59) patients (Kruskal-Wallis ANOVA followed by Dunn's test for multiple comparisons). (**B**) mRNA levels of glutamate turnover-related genes and glutamate ionotropic receptors subunits in atherosclerotic carotid plaques (n = 127, red) and non-atherosclerotic control arteries (n = 10, blue) from the BiKE cohort. *p < 0.05, **p < 0.01.

4.2.3 VSMCs express AMPA-type glutamate receptors in atherosclerotic plaques

In an effort to identify the cells expressing glutamate signaling-related transcripts, we turned to a scRNAseq dataset of human atherosclerotic coronary plaques. Key enzymes and receptors for glutamate synthesis, turnover, and signaling, were found in coronary atherosclerotic plaques. *GLS* was predominantly detected in endothelial cells, fibroblasts, differentiated VSMCs, modulated VSMCs, and plasma cells, while *GLUL* was primarily found in fibroblasts, macrophages, differentiated VSMCs, and pericytes[137]. Expression of the AMPA-type glutamate receptor subunits *GRIA1* and *GRIA*2 were exclusively detected in cell populations of mesenchymal origin, with the majority identified as VSMCs (**Figure 13**).

The ubiquitous expression of *GLS* and *GLUL* in human atherosclerotic lesions, and the reported abundance of glutamine[86], respectively their substrate and product, suggest that glutamate is in the atherosclerotic plaque. The concomitant expression of AMPA-type glutamate receptor subunits on VMSCs implies that glutamatergic signaling occurs inside atherosclerotic lesions.

As previously stated, lower *GRIA1* and *GRIA2* levels were found in symptomatic patients. Hence, glutamatergic signaling may be important in regulating the pathophysiology of atherosclerotic lesions.

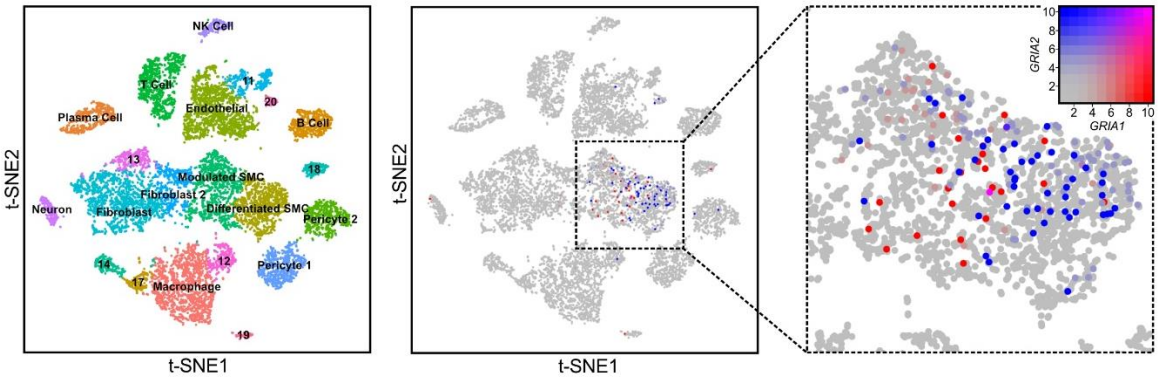

Figure 13: (left) tSNE visualization of single cell transcriptomic analysis of plaques (n = 5) derived from the right coronary artery of human patients (n = 4), colored according to broad cell clustering as indicated in the figure. Numbers denote unidentified clusters. NK, natural killer; SMC, smooth muscle cell. (right) tSNE visualization of single cell transcriptomic analysis of *GRIA1* (red) and *GRIA2* (blue) expression overlaid on the cell clusters from left. Colour legend indicates relative expression levels for *GRIA1* on x-axis and *GRIA2* on y-axis.

4.2.4 AMPA receptor modulation affects VSMC phenotype *in vitro*

Aimed to explore the function of AMPA receptors in VSMCs, we turned to human carotid SMCs (hcSMCs) culture. We assessed the presence of AMPA receptor proteins expression via immunofluorescence staining (**Figure 14A**). hcSMCs were then cultured in the presence or absence of NBQX, a known AMPA receptor antagonist[124]. Bulk RNA sequencing analysis showed significant differences in transcript levels of 464 genes between the two groups (**Figure 14B**). Interestingly, mRNA levels of the majority of the canonical markers for contractile VSMCs[82,84,138,139] were lower in cultures exposed to NBQX compared with vehicle. Gene ontology classification of the significantly differentially expressed genes revealed that specific pathways were affected, such as extracellular matrix organization, cell adhesion, and wound healing (**Figure 14C**).

These observations indicate that AMPA-type receptors in hcSMCs regulate expression of a gene set involved in phenotypic modulation, and possibly VSMC differentiation, promoting contractile features. Based on these findings, and the numerous reports linking the pathophysiology of atherosclerosis with VSMCs phenotype[81], it is important to investigate this biology further which may serve as a key for new therapeutical approaches for CVD.

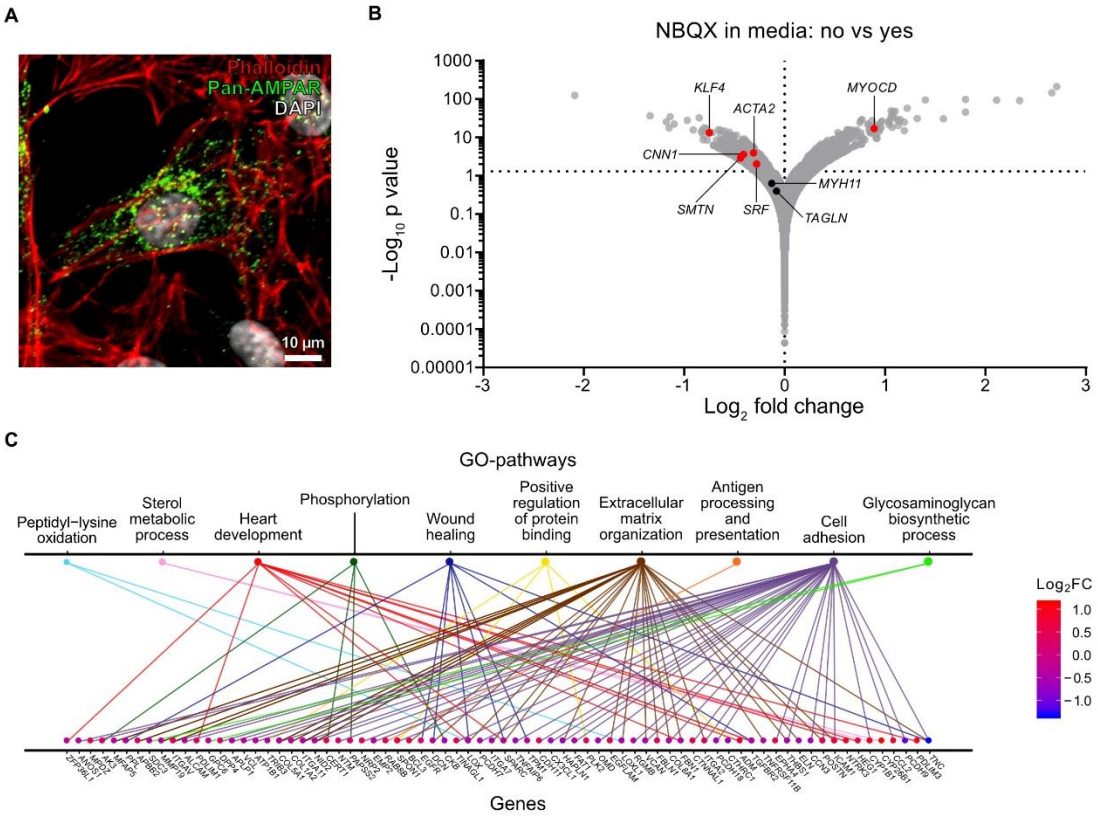

Figure 14: (**A**) hcSMCs were stained using anti-pan-AMPA receptor (pan-AMPAR) antibody (green), phalloidin (red), and DAPI (white). (**B**) Visualization of RNAseq data from hcSMCs grown in the presence (n = 3) or absence (n = 3) of the AMPAR antagonist NBQX for 24 h. Investigated SMC markers are shown in red when their expression is significantly different between conditions, otherwise in black. Actin alpha 2 (*ACTA2*), Kruppel-like factor 4 (*KLF4*), Myosin heavy chain 11 (*MYH11*), Myocardin (*MYOCD*), Calponin 1 (*CNN1*), Smoothelin (*SMTN*), Serum response factor (*SRF*), and Transgelin (*TAGLN*). (**C**) Distribution of the significantly regulated genes in the top 10 enriched Gene ontology (GO) pathways. The genes are color coded: genes labelled in blue are reduced, while red are increased in NBQX-treated hcSMCs.

4.3 PROJECT III

4.3.1 Batch-dependent inflammatory insult requires titration

It is known that there is a significant variation between batches of commercially available chemicals[140]. In mouse endotoxemia experiments, to evoke a consistent inflammatory insult within the physiological limits, a dose-response curve needs to be established for each LPS batch. In our results, LPS purchased from the same vendor but a different batch, has been used in endotoxemia experiments with a ~ 100-fold different concentration (8mg/kg – 0.1mg/kg)[112,141]. A dose-response curve was established as follows, mice were injected intraperitoneally with 0, 0.25, 0.5, 1, 2.5, 5, and 10 mg/kg of LPS and then euthanized using

carbon dioxide asphyxiation 90 minutes after injection. Previous observations indicate that serum TNF reaches its peak level between 60 and 90 min after injection[62]. Blood was collected and samples were incubated for 1 hour at room temperature and serum was retained. In this experiment, ELISA measurements of TNF levels showed a plateau at LPS doses above 2.5 mg/kg. Accordingly, 0.25 – 1 mg/kg of endotoxin was considered appropriate for studying inflammatory insults in a physiological range (**Figure 15**).

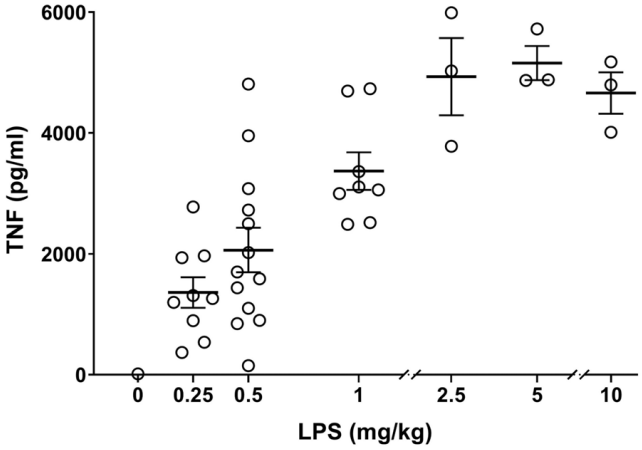

Figure 15: Serum TNF dose-response in endotoxemia. Mice were injected intraperitoneally with endotoxin at the reported concentrations, and blood was collected by cardiac puncture 90 min later. TNF was measured by ELISA (n = 3–13 mice per group).

4.3.2 Efficacious method for surgical isolation of the cervical left branch of the vagus nerve in mouse

Aimed to standardize an effective VNS practice in mice, we describe in detail the surgical procedure to isolate the vagus nerve for later analysis. Anesthesia was induced using isoflurane gas and mice were placed supine on a heating pad (**Figure 16A**), the neck area was shaved and swabbed with 70% ethanol. A ventral central cervical incision was made (**Figure 16B**). The salivary glands were exposed (**Figure 16C**) and separated to reveal the trachea (**Figure 16D**). Below the sternomastoid muscle can be found the carotid sheath, or neurovascular bundle (**Figure 16E**). The vagus nerve was isolated away from the carotid artery and the surrounding connective tissue (**Figure 16F**). A segment of silk strand for suture was placed under the nerve to facilitate electrode placement (**Figure 16G**).

Of note, minor trauma to the vagus nerve can easily occur during the surgical procedure and can significantly affect physiology, altering experimental outcome *per se*. Moreover, stretching the nerve during handling can cause stress that interferes with nerve function. This may result in evoking or impairing nerve activity, directly affecting the consistency of results between experiments

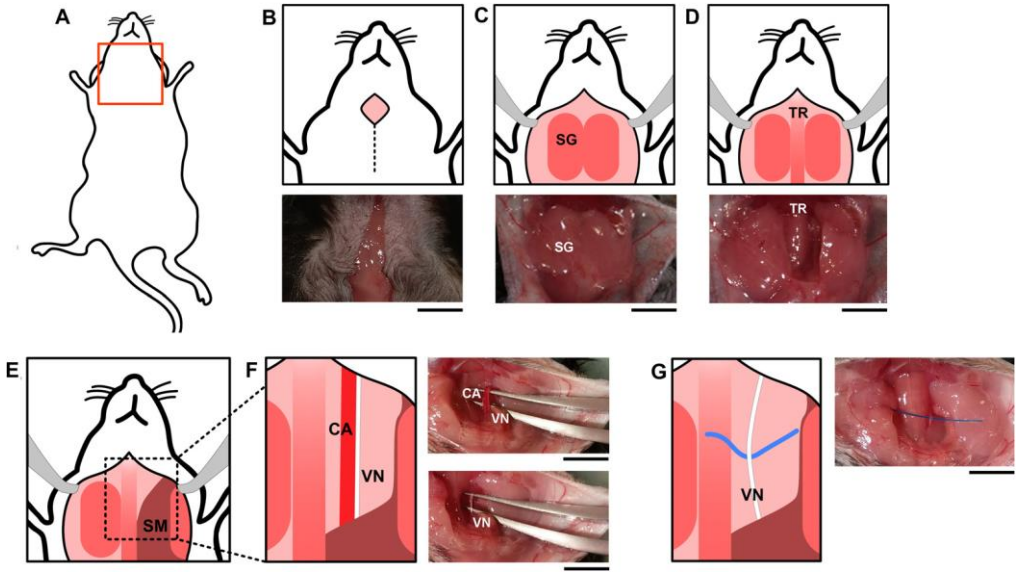

Figure 16: (**A**) The neck area of the mouse used for surgery. (**B**) The midline cervical incision made to expose the (**C**) salivary glands (SG) and (**D**) trachea (TR). Subcutaneous tissues between the (**E**) sternomastoid (SM) and sternohyoid muscles along the trachea were separated revealing the (**F**) common carotid artery (CA) and the cervical vagus nerve (VN). VN and CA are located parallel to each other and they need to be separated. (**G**) A piece of suture wire was placed under the nerve to facilitate electrode placement. The black scale bar indicates 5 mm.

4.3.3 Validated parameters for acute VNS in a mouse model of experimental inflammation

A constant current was used to induce nerve stimulation. In particular, a charge-balanced biphasic square waveform with an amplitude of 1 mA, 250 ms, and 50 ms interphase delay, at 10 Hz for 5 min was considered effective parameters (**Figure 17A**). It is known that delivering a charge-balanced pulse corresponds to minor tissue and electrode damage compared to unbalanced charges[142]. A constant current stimulator was used to maintain these parameters and an oscilloscope to visualize the voltage from the output and across the electrode-nerve interface (**Figures 17B, 17C**). The oscilloscope tracing was used to calculate the drop across the resistance. The delivery of consistent current requires that the electrical path is isolated from the surrounding tissues or extracellular liquid and specifically confined to the nerve. This was achieved by carefully suspending the nerve on the hook electrodes, avoiding mechanical injury that may cause adverse effects (**Figure 17D**).

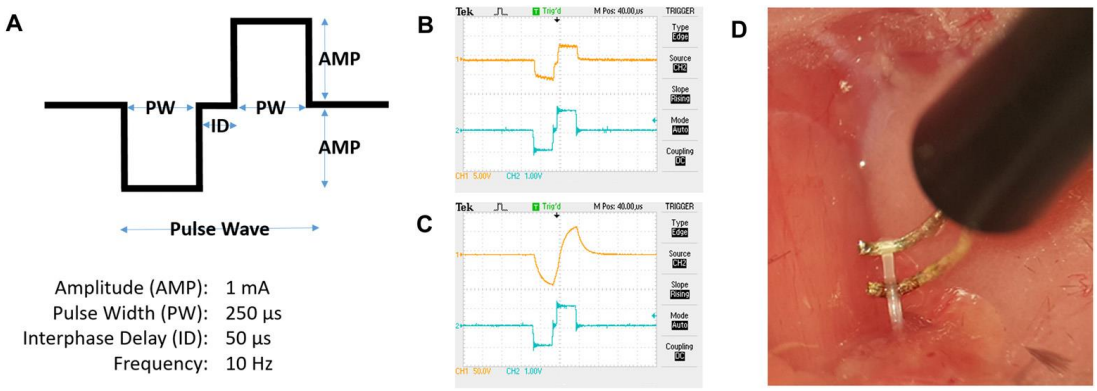

Figure 17: (**A**) Schematic representation of the pulse wave used for stimulation. (**B**) Oscilloscope tracing of voltage output from the digital to analog interface represented in blue (scale 1 V/square). Desired impedance and voltage measured over the electrode are in orange (scale 5 V/square). (**C**) Tracing of electrode-nerve interface with a high impedance (orange tracing, scale 50 V/square). (**D**) The isolated vagus nerve segment placed on a hook electrode.

4.4 PROJECT IV

4.4.1 Electrical VNS accelerates resolution onset and increases efferocytosis in experimental peritonitis

To investigate the effect of VNS on inflammation resolution, we turned to a mouse model of zymosan-induced peritonitis. The inflammatory stimulus was administrated after cervical VNS and peritoneal exudates were collected at consecutive time points. Flow cytometry was used to establish infiltrating neutrophil numbers at 0, 4, 12, 24, 36, 48 hours, indicating the course of inflammation. However, the peak of neutrophil count may be not easily identified, due to the limited number of timepoints. Moreover, these experiments are characterized by a large variation in the numbers of neutrophils, making interpretation of the resolution time challenging[143]. In our view, the currently used method to measure resolution time[129] does not fully capture the dynamics of inflammation decay. For this reason, we established a mathematical method to calculate inflammation decay and derive the resolution interval (Ri). We used a system of ODEs $\dot{x}(t) = f(x(t))$, which showed a 34% reduction in VNS-treated mice compared to sham (**Figure 18A**). We observed that the decrease in neutrophil count, used as an indicator of the inflammation decay, was exponential (**Figure 18B)**. This observation was validated in several publicly available datasets of zymosan-induced peritonitis in wild-type mice. The exponential decay model was used to calculate the resolution rate, in which the slope represents the inflammation decay (I_d), which was significantly higher in the VNS-treated group (**Figure 18C**).

A key process of inflammation resolution is efferocytosis, which represents the clearance of cellular debris and apoptotic neutrophils at the site of the insult. Hence, we next investigated

VNS effects on MΦ-mediated clearance of neutrophils in the peritoneal exudates collected 12 hours after zymosan injection. We immunofluorescently stained for F4/80, a known marker of MΦ, and Ly6G, to identify neutrophils. Co-localization of the signal was considered an indication of active efferocytosis (**Figure 18D**). Exudates from VNS-treated mice showed a significantly higher number of $F4/80^{+}Ly6G^{+}$ cells in flow cytometry analysis, as compared with sham (**Figure 18E**).

These observations appear to confirm that VNS has significant effects in both phases of the typical inflammatory response, regulating the inflammation onset via infiltrating neutrophil numbers, and resolution via efferocytosis.

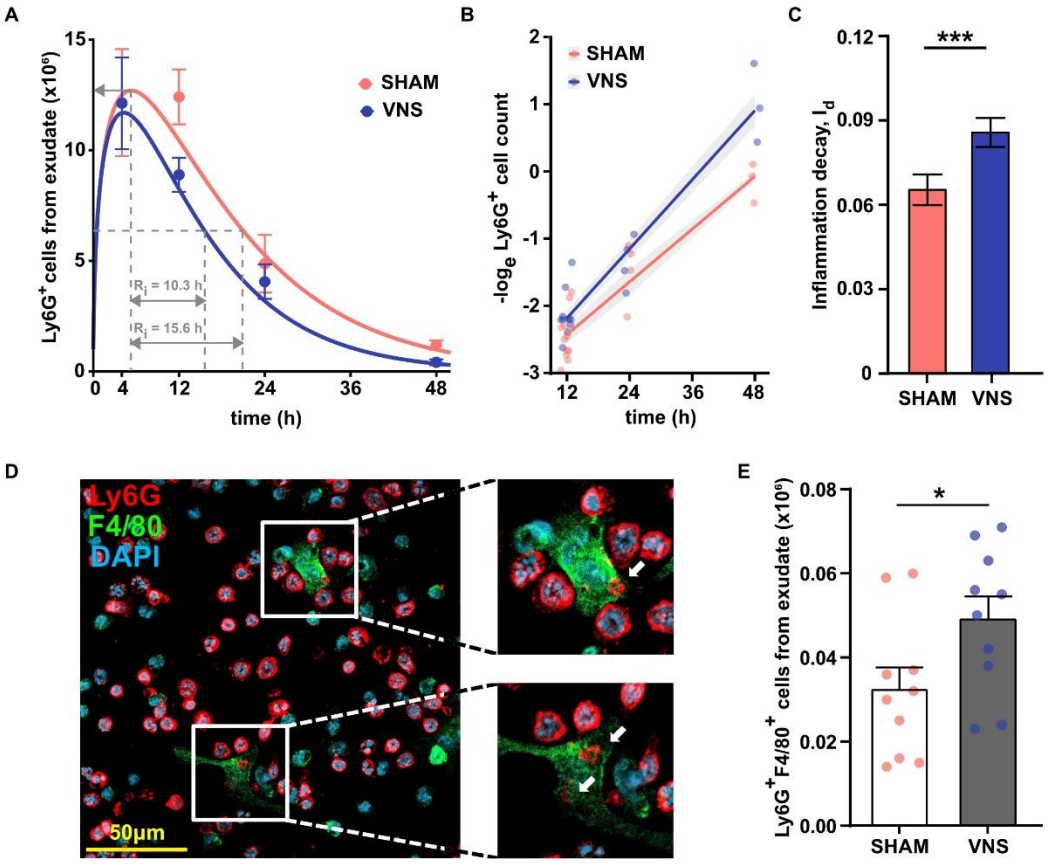

Figure 18: (**A**) Numbers of $Ly6G^{+}$ cell at the indicated timepoints were modelled using ODE model. The resolution interval (Ri) corresponds to the time interval between peak of neutrophils count to its halving. (**B**) Logarithmic cell counts from (A) and linear regression analysis with areas of mean squared error represented in gray. (**C**) Inflammation decay (Id) from (B) (Student's t-test; n = 2-5 experiments). (**D**) Immunofluorescence staining of adherent cells from peritoneal exudate. White arrows indicate $Ly6G^{+}$ regions within $F4/80^{+}$ cells collected at 12 h after zymosan-induced peritonitis. (**G**) Flow cytometry quantification of absolute numbers of $F480^{+}Ly6G^{+}$ cells collected at 12 h after zymosan-induced peritonitis (Student's t-test). *p < 0.05, ***p <0.001.

4.4.2 VNS enhances endogenous SPM production *in vivo*

SPMs directly promote efferocytosis and counter-balance pro-inflammatory cytokines. Since SPMs can be considered as a driving force in the resolution of inflammation, we investigated whether VNS regulates their biosynthesis. In the previously described peritonitis mouse model, levels of peritoneal lipid mediator were determined via liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based LM metabololipidomics. We identified mediators from all the major SPM families, which include DHA, EPA, DPA, and AA. Levels of DHA-derived resolvins, protectins, and maresins, as well as DPA-derived protectins, were all significantly higher in the VNS-treated group. No significant difference was observed regarding EPA- or AA-derived SPM between VNS- and sham-treated groups. Importantly, no difference was observed in pro-inflammatory lipid mediators (**Figure 19**).

These findings indicate that VNS-treatment promotes a pro-resolving state via increasing specific SPM families, which suggests a direct regulation on Arachidonate 15-Lipoxygenase (Alox15) activity, the enzyme responsible for their biosynthesis.

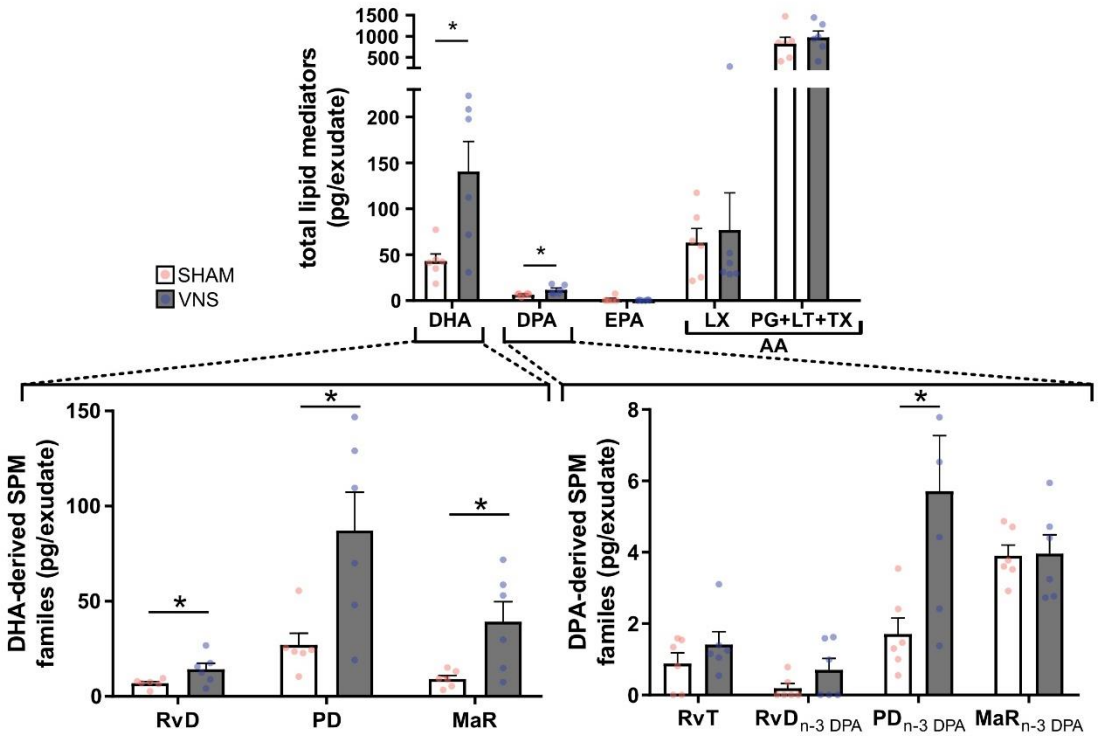

Figure 19: LC-MS/MS based profiling of lipid mediators in peritoneal exudates collected 12 hours after intraperitoneal zymosan injection. Lipid mediators identified were from the major bioactive metabolomes DHA, EPA, DPA, and AA. Levels of identified DHA-derived and DPA-derived SPM families in peritoneal exudates were significantly different between VNS and sham groups (n = 2; Student's t-test). *$p < 0.05$

4.4.3 Alox15 and α7nAChR participate in VNS-enhanced resolution of inflammation

To investigate the relevance of Alox15 in the VNS-regulated inflammation resolution, we used Alox15-deficient mice for zymosan-induced peritonitis. After administration of the insult, peritoneal exudates were collected, and infiltrating neutrophils were counted, at different timepoints (**Figure 20A**). The logarithmic conversion of the exponential decay model was used to calculate I_d (**Figure 20B**), which showed no significant difference between VNS- and sham groups (**Figure 20C**). Flow cytometry analysis of $Ly6G^+$ neutrophils, collected 12 hours after the zymosan injection, indicated no significant difference between groups (**Figure 20D**). These observations show that in the absence of Alox15, VNS treatment failed to significantly affect the resolution of inflammation.

There are several reported observations linking SPM and ACh. For example, ACh upregulates the Alox15-derived 17-hydroxy-DHA in lymphoid cells, and disruption of the vagus nerve corresponds to a decrease of peritoneal ACh, which is associated with changes in the lipid mediators profile[143]. It is not yet fully elucidated the mechanism for these effects, however, it is known that α7nAChR is expressed on immune cells and is a key component of the inflammatory reflex[71]. To investigate the relevance of α7nAChR and the inflammatory reflex in the context of VNS-mediated control of inflammation in zymosan-induced peritonitis, we turned to α7nAChR-deficient mice deficient. We analyzed neutrophil numbers in the peritoneal cavity at different time points after the administration of the insult (**Figure 20E**). We did not observe a significant difference in the inflammation I_d between VNS- or sham-treated groups (**Figure 20F, 20G**). The numbers of $Ly6G^+$ cells in peritoneal exudates after 12 hours from zymosan injection were similar between groups (**Figure 20H**). Hence, α7nAChR was essential for VNS-mediated regulation of the inflammation resolution rate.

Together, these experiments support the notion that Alox15-derived SPMs are important mediators in the inflammation decay processes, which are significantly affected by signals carried by the vagus nerve, in this model.

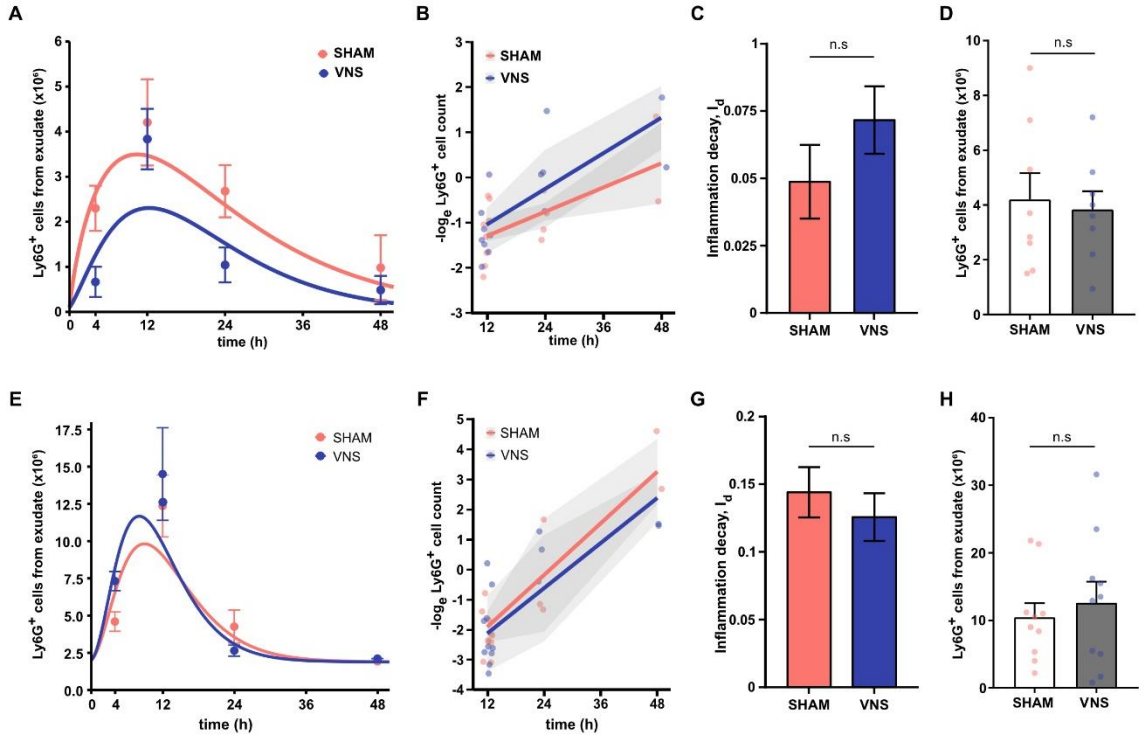

Figure 20: (**A**) Numbers of Ly6G$^+$ cells in Alox15 deficient mice at the indicated timepoints were modelled using ODE model. (**B**) Logarithmic cell counts from (A) and linear regression analysis with areas of mean squared error represented in gray. (**C**) Inflammation decay (I_d) from (B) (Student's t-test; n = 2 experiments). (**D**) Flow cytometry quantification of peritoneal exudate Ly6G$^+$ cells in VNS- and sham-treated groups at 12 hours after zymosan-injection. (**E**) Numbers of Ly6G$^+$ cells in α7nAChR deficient mice at the indicated timepoints were modelled using ODE model. (**F**) Logarithmic cell counts from (E) and linear regression analysis with areas of mean squared error represented in gray. (**G**) Inflammation decay (I_d) from (F) (Student's t-test; n = 2 experiments). (**H**) Flow cytometry quantification of peritoneal exudate Ly6G$^+$ cells in VNS- and sham-treated groups at 12 hours after zymosan-injection. Student's t-test; n.s. = non-significant.

4.5 PROJECT V

4.5.1 TINS evokes motor responses at the envelope frequency

Aimed to investigate whether TINS can stimulate a peripheral nerve in mice, we performed a TI-mediated sciatic nerve stimulation. We used two separate pairs of stimulating electrodes, controlled by two bipolar constant-current stimulators, to increase spatial resolution and profundity of the envelope of frequencies (**Figure 21A**). We delivered a current of 350 μA within 3 kHz carriers. The frequency offset ranges spanned from 0.5 Hz to 4 Hz. We observed a defined and regular contraction of the muscle and limb movements at intervals corresponding to the envelope frequency (**Figure 2B**).

Traditional TENS was attempted as control. We used two stimulating electrodes and the same parameters used for TINS, however no sciatic nerve stimulation effects were observed (**Figure 21C**). Muscle movement was evoked with TENS only using currents higher than1 mA.

Of note, the TINS method was effective but the current set-up needs special care. It is fundamental to place the electrodes in line with the sciatic nerve, to overlap the envelope directly with the nerve. Novel approaches based on flexible grid-electrodes are currently being explored. Once optimized, this will allow us to avoid manual adjustments of the electrodes, and remotely change the configuration of the grid.

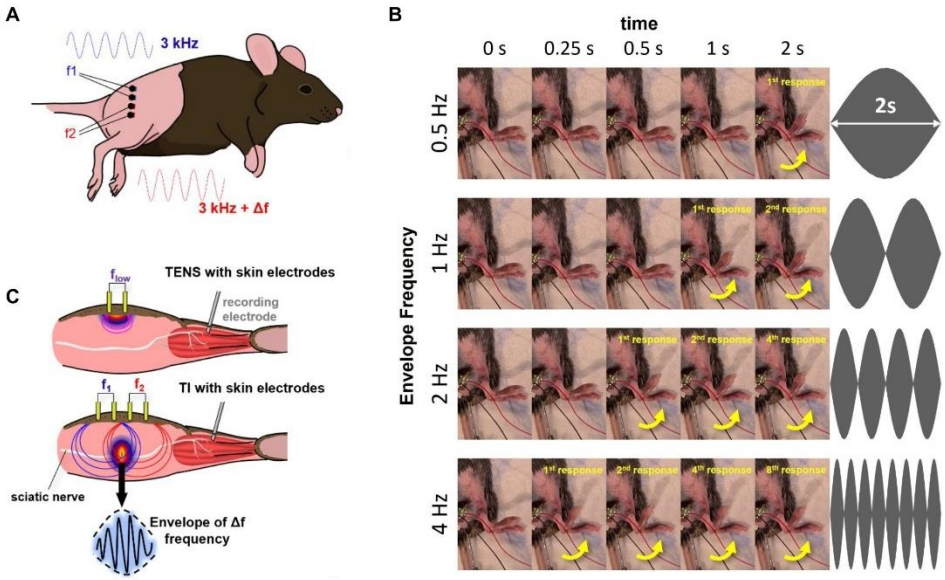

Figure 21: (**A**) TINS method requires two pairs of electrodes stimulated at high frequency (3 kHz) and a frequency offset (Δf). The envelope of Δf is the frequency that evoke nerve activity at the focal point, where the two electric fields interfere. (**B**) Carrier frequencies of 3 kHz, Δf and 350 μA are applied above the sciatic nerve. Δf is increased from 0.5 Hz to 4 Hz and the nerve stimulation-dependent response (leg movement) follows the envelope frequency. (**C**) TINS allows reaching targets deeper below the skin than transcutaneous electrical nerve stimulation TENS.

5 CONCLUSION

In **Project I**, for the first time in humans, we identified functional $ChAT^{+}CD4^{+}$ T cells. We characterized these cells, and showed that T cell activation promoted increased levels of ChAT mRNA. We measured their release of ACh and investigated their role in arterial relaxation *in vitro* and *ex vivo*. We found $ChAT^{+}$ T cells in blood collected from patients in circulatory distress and observed that their frequency correlated with survival. The observations we reported in this study represent the groundwork for new studies in humans, by which new mechanisms of nerve-immune cell-tissue interactions may be identified. Hence, the possibility to design novel therapies targeting extravasation of immune cells and local perfusion at target sites.

In **Project II**, we provided a comprehensive map of nervous system-associated signaling components expressed in atherosclerotic plaques. This analysis segregated components of the glutamatergic axis, in particular the glutamate AMPA-type receptor subunits. We thus found components of neurotransmitter signaling in human atherosclerosis, observed an association between low glutamate-receptor expression and adverse clinical events, and found that glutamate signaling regulates smooth muscle cell phenotypic modulation *in vitro*. This work lays the foundation for further mechanistic studies and exploration of the roles of neurotransmitter signaling in regulation of chronic vascular inflammation aiming to improve the understanding of fundamental vascular biology in health and disease.

The work carried out in **Project III** represents our attempt to share knowledge of electrical vagus nerve stimulation in experimental mice to facilitate reproducible experimentation. In our experience, several elements are key for consistent and reproducible results in endotoxemia mouse experiments, such as insult titration, electrode fabrication, surgical isolation of the nerve, sample handling, and stimulation parameters. Here we established and described a protocol for an effective methodology for acute cervical VNS in an endotoxemia mouse model.

In **Project IV**, we provide evidence that electrical activation of the cervical vagus nerve accelerates inflammation resolution in mice through a cholinergic mechanism that involves synthesis of specialized pro-resolving mediators. We modeled the kinetics of inflammation using available data and established mathematical analysis for calculating the speed of inflammation decay. We observed that VNS accelerated resolution of inflammation, promoted efferocytosis and increased Alox15-derived SPM levels, shifting the lipid mediator balance towards an enhanced pro-resolving profile. The effect of VNS observed in wild-type mice was disrupted in mice deficient in either Alox15 or the ACh receptor α7nAChR, a key component of the afferent branch of the inflammatory reflex. Together, this work provides evidence that the vagus nerve activity controls the inflammation microenvironment and SPM biosynthesis, hence directly affecting the resolution of inflammation.

Ultimately, **Project V**, represents our efforts to overcome limitations in the methodologies of peripheral nerve stimulation, particularly the lack of anatomical specificity inherent with the available non-invasive methods. As a proof of concept, we successfully used a non-invasive TI-based stimulation method to selectively activate the sciatic nerve in mice. These preliminary results lay the groundwork for effective, nerve-selective and non-invasive peripheral nerve stimulation. Currently, grids of electrodes adherent to the skin are being developed in an attempt to permit implementation of more complex stimulation patterns for increased spatial resolution. Once this method is properly established, there are likely innumerable applications in bioelectronic medicine and research, e.g. chronic VNS in models of non-resolving inflammatory diseases.

In summary, this thesis studies aspects of neural signaling in inflammation and reveal new details on glutamatergic and cholinergic signals in inflammation and vascular pathophysiology. The work also contributes new methodology which we postulate will be helpful in further mapping of the neural signals that regulate inflammation and for clinical translation of discoveries in this field.

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7 REFERENCES

1. Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ. Rethinking inflammation: neural circuits in the regulation of immunity. *Immunol Rev*. 2012;248(1):188-204. doi:10.1111/j.1600-065X.2012.01138.x

2. Andersson U, Tracey KJ. Reflex Principles of Immunological Homeostasis. *Annu Rev Immunol*. 2012;30(1):313-335. doi:10.1146/annurev-immunol-020711-075015

3. Borovikova L V., Ivanova S, Zhang M, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature*. 2000;405(6785):458-462. doi:10.1038/35013070

4. Inoue T, Abe C, Sung SJ, et al. Vagus nerve stimulation mediates protection from kidney ischemia-reperfusion injury through α7nAChR+ splenocytes. *J Clin Invest*. 2016;126(5):1939-1952. doi:10.1172/JCI83658

5. Kwan H, Garzoni L, Liu HL, et al. Vagus Nerve Stimulation for Treatment of Inflammation: Systematic Review of Animal Models and Clinical Studies. *Bioelectron Med*. 2016;3:1-6.

6. Eberhardson M, Tarnawski L, Centa M, Olofsson PS. Neural Control of Inflammation: Bioelectronic Medicine in Treatment of Chronic Inflammatory Disease. *Cold Spring Harb Perspect Med*. 2020;10(3):a034181. doi:10.1101/cshperspect.a034181

7. Gisterå A, Hansson GK. The immunology of atherosclerosis. *Nat Rev Nephrol*. 2017;13(6):368-380. doi:10.1038/nrneph.2017.51

8. Libby P, Buring JE, Badimon L, et al. Atherosclerosis. *Nat Rev Dis Prim*. 2019;5(1):56. doi:10.1038/s41572-019-0106-z

9. Caravaca AS, Centa M, Gallina AL, Tarnawski L, Olofsson PS. Neural reflex control of vascular inflammation. *Bioelectron Med*. 2020;6(1):3. doi:10.1186/s42234-020-0038-7

10. Furchgott RF, Zawadzki J V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980. doi:10.1038/288373a0

11. Tracey KJ. The inflammatory reflex. *Nature*. 2002;420(6917):853-859. doi:10.1038/nature01321

12. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest*. 2007;117(2):289-296. doi:10.1172/JCI30555

13. Andersson U, Tracey KJ. Neural reflexes in inflammation and immunity. *J Exp Med*. 2012;209(6):1057-1068. doi:10.1084/jem.20120571

14. Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1β generation. *Clin Exp Immunol*. 2007;147(2):227-235. doi:10.1111/j.1365-2249.2006.03261.x

15. Pober JS, Sessa WC. Inflammation and the blood microvascular system. *Cold Spring Harb Perspect Biol*. 2015;7(1). doi:10.1101/cshperspect.a016345

16. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol*. 2008;8(5):349-361. doi:10.1038/nri2294

17. Sansbury BE, Spite M. Resolution of Acute Inflammation and the Role of Resolvins in Immunity, Thrombosis, and Vascular Biology. *Circ Res*. 2016;119(1):113-130. doi:10.1161/CIRCRESAHA.116.307308

18. Ortega-Gómez A, Perretti M, Soehnlein O. Resolution of inflammation: An integrated view. *EMBO Mol Med*. 2013;5(5):661-674. doi:10.1002/emmm.201202382

19. Basil MC, Levy BD. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nat Rev Immunol*. 2016;16(1):51-67. doi:10.1038/nri.2015.4

20. Nathan C, Ding A. Nonresolving Inflammation. *Cell*. 2010;140(6):871-882. doi:10.1016/J.CELL.2010.02.029

21. Zhang J-M, An J. Cytokines, inflammation, and pain. *Int Anesthesiol Clin*. 2007;45(2):27-37. doi:10.1097/AIA.0b013e318034194e

22. Placek K, Schultze JL, Aschenbrenner AC. Epigenetic reprogramming of immune cells in injury, repair, and resolution. *J Clin Invest*. 2019;129(8):2994-3005. doi:10.1172/JCI124619

23. Johnstone J, Loeb M. Inlammatory bowel disease. In: *Primary Care Management of Community-Acquired Pneumonia*. Vol 347. Unitec House, 2 Albert Place, London N3 1QB, UK: Future Medicine Ltd; 2011:10-19. doi:10.2217/ebo.11.37

24. Smolen JS, Aletaha D, Barton A, et al. Rheumatoid arthritis. *Nat Rev Dis Prim*. 2018;4(1):18001. doi:10.1038/nrdp.2018.1

25. Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther*. 2008;117(2):244-279. doi:10.1016/j.pharmthera.2007.10.001

26. Scheinecker C, Redlich K, Smolen JS. Cytokines as Therapeutic Targets: Advances and Limitations. *Immunity*. 2008;28(4):440-444. doi:10.1016/j.immuni.2008.03.005

27. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. *Nat Immunol*. 2005;6(12):1191-1197. doi:10.1038/ni1276

28. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature*. 2014;510(7503):92-101. doi:10.1038/nature13479

29. Buckley CD, Gilroy DW, Serhan CN. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. *Immunity*. 2014;40(3):315-327. doi:10.1016/j.immuni.2014.02.009

30. Serhan CN, Chiang N, Dalli J, Levy BD. Lipid mediators in the resolution of inflammation. *Cold Spring Harb Perspect Biol*. 2015;7(2). doi:10.1101/cshperspect.a016311

31. Lawrence T, Gilroy DW. Chronic inflammation: A failure of resolution? *Int J Exp*

Pathol. 2007;88(2):85-94. doi:10.1111/j.1365-2613.2006.00507.x

32. Serhan CN. Novel lipid mediators and resolution mechanisms in acute inflammation: To resolve or not? *Am J Pathol*. 2010;177(4):1576-1591. doi:10.2353/ajpath.2010.100322

33. Furman D, Campisi J, Verdin E, et al. Chronic inflammation in the etiology of disease across the life span. *Nat Med*. 2019;25(12):1822-1832. doi:10.1038/s41591-019-0675-0

34. Sundman E, Olofsson PS. Neural control of the immune system. *Adv Physiol Educ*. 2015;38(2):135-139. doi:10.1152/advan.00094.2013

35. Tracey KJ. Reflex control of immunity. *Nat Rev Immunol*. 2009;9(6):418-428. doi:10.1038/nri2566

36. Huang S, Ziegler CGK, Austin J, et al. Lymph nodes are innervated by a unique population of sensory neurons with immunomodulatory potential. *Cell*. 2021;184(2):441-459.e25. doi:10.1016/j.cell.2020.11.028

37. Goehler LE, Gaykema RPA, Hansen MK, Anderson K, Maier SF, Watkins LR. Vagal immune-to-brain communication: a visceral chemosensory pathway. *Auton Neurosci*. 2000;85(1-3):49-59. doi:10.1016/S1566-0702(00)00219-8

38. Sun J, Singh V, Kajino-Sakamoto R, Aballay A. Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes. *Science (80-).* 2011;332(6030):729-732. doi:10.1126/science.1203411

39. Tracey KJ. Ancient neurons regulate immunity. *Science (80-).* 2011;332(6030):673-674. doi:10.1126/science.1206353

40. Liu Y, Sellegounder D, Sun J. Neuronal GPCR OCTR-1 regulates innate immunity by controlling protein synthesis in Caenorhabditis elegans. *Sci Rep*. 2016;6(1):36832. doi:10.1038/srep36832

41. Tarnawski L, Olofsson PS. Inflammation neuroscience: neuro-immune crosstalk and interfaces. *Clin Transl Immunol*. 2021;10(11):1-11. doi:10.1002/cti2.1352

42. Pinho-Ribeiro FA, Verri WA, Chiu IM. Nociceptor Sensory Neuron–Immune Interactions in Pain and Inflammation. *Trends Immunol*. 2017;38(1):5-19. doi:10.1016/j.it.2016.10.001

43. Chiu IM, Heesters BA, Ghasemlou N, et al. Bacteria activate sensory neurons that modulate pain and inflammation. *Nature*. 2013;501(7465):52-57. doi:10.1038/nature12479

44. Fernandes ES, Liang L, Smillie S-J, et al. TRPV1 deletion enhances local inflammation and accelerates the onset of systemic inflammatory response syndrome. *J Immunol*. 2012;188(11):5741-5751. doi:10.4049/jimmunol.1102147

45. Pongratz G, Straub RH. Role of peripheral nerve fibres in acute and chronic inflammation in arthritis. *Nat Rev Rheumatol*. 2013;9(2):117-126. doi:10.1038/nrrheum.2012.181

46. Hodo TW, de Aquino MTP, Shimamoto A, Shanker A. Critical Neurotransmitters in the Neuroimmune Network. *Front Immunol*. 2020;11(August):1869. doi:10.3389/fimmu.2020.01869

47. Kerage D, Sloan EK, Mattarollo SR, McCombe PA. Interaction of neurotransmitters and neurochemicals with lymphocytes. *J Neuroimmunol*. 2019;332(March):99-111. doi:10.1016/j.jneuroim.2019.04.006

48. Rosas-Ballina M, Olofsson PS, Ochani M, et al. Acetylcholine-Synthesizing T Cells Relay Neural Signals in a Vagus Nerve Circuit. *Science (80-)*. 2011;334(6052):98-101. doi:10.1126/science.1209985

49. Andersson J. The inflammatory reflex - Introduction. *J Intern Med*. 2005;257(2):122-125. doi:10.1111/j.1365-2796.2004.01440.x

50. Thompson N, Mastitskaya S, Holder D. Avoiding off-target effects in electrical stimulation of the cervical vagus nerve: Neuroanatomical tracing techniques to study fascicular anatomy of the vagus nerve. *J Neurosci Methods*. 2019. doi:10.1016/j.jneumeth.2019.108325

51. Lewis SM, Williams A, Eisenbarth SC. Structure and function of the immune system in the spleen. *Sci Immunol*. 2019;4(33):eaau6085. doi:10.1126/sciimmunol.aau6085

52. Breit S, Kupferberg A, Rogler G, Hasler G. Vagus Nerve as Modulator of the Brain–Gut Axis in Psychiatric and Inflammatory Disorders. *Front Psychiatry*. 2018;9(MAR). doi:10.3389/fpsyt.2018.00044

53. Bonaz B, Sinniger V, Pellissier S. The vagus nerve in the neuro-immune axis: Implications in the pathology of the gastrointestinal tract. *Front Immunol*. 2017;8(NOV). doi:10.3389/fimmu.2017.01452

54. Fairchild KD, Srinivasan V, Randall Moorman J, Gaykema RPA, Goehler LE. Pathogen-induced heart rate changes associated with cholinergic nervous system activation. *Am J Physiol Integr Comp Physiol*. 2011;300(2):R330-R339. doi:10.1152/ajpregu.00487.2010

55. Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. In: *Autonomic Neuroscience: Basic and Clinical*. Vol 85. ; 2000:1-17. doi:10.1016/S1566-0702(00)00215-0

56. Watkins LR, Goehler LE, Relton JK, et al. Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. *Neurosci Lett*. 1995;183(1-2):27-31. doi:10.1016/0304-3940(94)11105-R

57. Niijima A. The afferent discharges from sensors for interleukin 1 beta in the hepatoportal system in the anesthetized rat. *J Auton Nerv Syst*. 1996;61(3):287-291. doi:10.1016/s0165-1838(96)00098-7

58. Mirakaj V, Dalli J, Granja T, Rosenberger P, Serhan CN. Vagus nerve controls resolution and pro-resolving mediators of inflammation. *J Exp Med*. 2014;211(6):1037-1048. doi:10.1084/jem.20132103

59. Mirakaj V, Thix CA, Laucher S, et al. Netrin-1 dampens pulmonary inflammation

during acute lung injury. *Am J Respir Crit Care Med*. 2010;181(8):815-824. doi:10.1164/rccm.200905-0717OC

60. Ranganathan P V., Jayakumar C, Mohamed R, Dong Z, Ramesh G. Netrin-1 regulates the inflammatory response of neutrophils and macrophages, and suppresses ischemic acute kidney injury by inhibiting COX-2-mediated PGE2 production. *Kidney Int*. 2013;83(6):1087-1098. doi:10.1038/ki.2012.423

61. Bratton BO, Martelli D, McKinley MJ, Trevaks D, Anderson CR, McAllen RM. Neural regulation of inflammation: no neural connection from the vagus to splenic sympathetic neurons. *Exp Physiol*. 2012;97(11):1180-1185. doi:10.1113/expphysiol.2011.061531

62. Huston JM, Ochani M, Rosas-Ballina M, et al. Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. *J Exp Med*. 2006;203(7):1623-1628. doi:10.1084/jem.20052362

63. Rosas-Ballina M, Ochani M, Parrish WR, et al. Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. *Proc Natl Acad Sci U S A*. 2008;105(31):11008-11013. doi:10.1073/pnas.0803237105

64. Bassi GS, Kanashiro A, Coimbra NC, Terrando N, Maixner W, Ulloa L. Anatomical and clinical implications of vagal modulation of the spleen. *Neurosci Biobehav Rev*. 2020;112:363-373. doi:10.1016/j.neubiorev.2020.02.011

65. Murray K, Rude KM, Sladek J, Reardon C. Divergence of neuroimmune circuits activated by afferent and efferent vagal nerve stimulation in the regulation of inflammation. *J Physiol*. February 2021:JP281189. doi:10.1113/JP281189

66. Olofsson PS, Steinberg BE, Sobbi R, et al. Blood pressure regulation by CD4+ lymphocytes expressing choline acetyltransferase. *Nat Biotechnol*. 2016;34(10):1066-1071. doi:10.1038/nbt.3663

67. Cox MA, Duncan GS, Lin GHY, et al. Choline acetyltransferase–expressing T cells are required to control chronic viral infection. *Science (80-)*. 2019. doi:10.1126/science.aau9072

68. de Jonge WJ, van der Zanden EP, The FO, et al. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol*. 2005;6(8):844-851. doi:10.1038/ni1229

69. Lu B, Kwan K, Levine YA, et al. α7 nicotinic acetylcholine receptor signaling inhibits inflammasome activation by preventing mitochondrial DNA release. *Mol Med*. 2014;20(1):350-358. doi:10.2119/molmed.2013.00117

70. Vieira-Alves I, Coimbra-Campos LMC, Sancho M, da Silva RF, Cortes SF, Lemos VS. Role of the α7 Nicotinic Acetylcholine Receptor in the Pathophysiology of Atherosclerosis. *Front Physiol*. 2020;11(December):1-8. doi:10.3389/fphys.2020.621769

71. Wang H, Yu M, Ochani M, et al. Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation. *Nature*. 2003;421(6921):384-388. doi:10.1038/nature01339

72. Olofsson PS, Katz DA, Rosas-Ballina M, et al. α7 nicotinic acetylcholine receptor (α7nAChR) expression in bone marrow-derived non-T cells is required for the inflammatory reflex. *Mol Med*. 2012;18(1):539-543. doi:10.2119/molmed.2011.00405

73. van Westerloo DJ, Giebelen IA, Florquin S, et al. The Vagus Nerve and Nicotinic Receptors Modulate Experimental Pancreatitis Severity in Mice. *Gastroenterology*. 2006;130(6):1822-1830. doi:10.1053/j.gastro.2006.02.022

74. Pavlov VA, Ochani M, Yang LH, et al. Selective α7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. *Crit Care Med*. 2007;35(4):1139-1144. doi:10.1097/01.CCM.0000259381.56526.96

75. Rana M, Fei-Bloom Y, Son M, et al. Constitutive Vagus Nerve Activation Modulates Immune Suppression in Sepsis Survivors. *Front Immunol*. 2018;9(SEP):1-9. doi:10.3389/fimmu.2018.02032

76. Miller LE, Jüsten H, Schcölmerich J, Straub RH. The loss of sympathetic nerve fibers in the synovial tissue of patients with rheumatoid arthritis is accompanied by increased norepinephrine release from synovial macrophages. *FASEB J*. 2000;14(13):2097-2107. doi:10.1096/fj.99-1082com

77. Straub RH. Pathogenesis and Neuroendocrine Immunology. In: *The Origin of Chronic Inflammatory Systemic Diseases and Their Sequelae*. Elsevier; 2015:59-129. doi:10.1016/B978-0-12-803321-0.00002-1

78. Riol-Blanco L, Ordovas-Montanes J, Perro M, et al. Nociceptive sensory neurons drive interleukin-23-mediated psoriasiform skin inflammation. *Nature*. 2014;510(7503):157-161. doi:10.1038/nature13199

79. Ruparelia N, Chai JT, Fisher EA, Choudhury RP. Inflammatory processes in cardiovascular disease: a route to targeted therapies. *Nat Rev Cardiol*. 2017;14(3):133-144. doi:10.1038/nrcardio.2016.185

80. Timmis A, Townsend N, Gale C, et al. European Society of Cardiology: Cardiovascular Disease Statistics 2017. *Eur Heart J*. 2018;39(7):508-579. doi:10.1093/eurheartj/ehx628

81. Bennett MR, Sinha S, Owens GK. Vascular Smooth Muscle Cells in Atherosclerosis. *Circ Res*. 2016;118(4):692-702. doi:10.1161/CIRCRESAHA.115.306361

82. Gomez D, Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res*. 2012;95(2):156-164. doi:10.1093/cvr/cvs115

83. Gomez D, Owens GK. Reconciling Smooth Muscle Cell Oligoclonality and Proliferative Capacity in Experimental Atherosclerosis. *Circ Res*. 2016;119(12):1262-1264. doi:10.1161/CIRCRESAHA.116.310104

84. Liu M, Gomez D. Smooth Muscle Cell Phenotypic Diversity. *Arterioscler Thromb Vasc Biol*. 2019;39(9):1715-1723. doi:10.1161/ATVBAHA.119.312131

85. Allahverdian S, Chaabane C, Boukais K, Francis GA, Bochaton-Piallat M-L. Smooth muscle cell fate and plasticity in atherosclerosis. *Cardiovasc Res*. 2018;114(4):540-550. doi:10.1093/cvr/cvy022

86. Curi R. Glutamine, gene expression, and cell function. *Front Biosci*. 2007;12(1):344. doi:10.2741/2068

87. Yelamanchi SD, Jayaram S, Thomas JK, et al. A pathway map of glutamate metabolism. *J Cell Commun Signal*. 2016;10(1):69-75. doi:10.1007/s12079-015-0315-5

88. Niciu MJ, Kelmendi B, Sanacora G. Overview of glutamatergic neurotransmission in the nervous system. *Pharmacol Biochem Behav*. 2012;100(4):656-664. doi:10.1016/j.pbb.2011.08.008

89. Skerry TM, Genever PG. Glutamate signalling in non-neuronal tissues. *Trends Pharmacol Sci*. 2001;22(4):174-181. doi:10.1016/S0165-6147(00)01642-4

90. Gill SS, Pulido OM, Mueller RW, McGuire PF. Molecular and immunochemical characterization of the ionotropic glutamate receptors in the rat heart. *Brain Res Bull*. 1998;46(5):429-434. doi:10.1016/S0361-9230(98)00012-4

91. Dumas SJ, Bru-Mercier G, Courboulin A, et al. NMDA-Type Glutamate Receptor Activation Promotes Vascular Remodeling and Pulmonary Arterial Hypertension. *Circulation*. 2018;137(22):2371-2389. doi:10.1161/CIRCULATIONAHA.117.029930

92. Qi L, Qi Q, Prudente S, et al. Association Between a Genetic Variant Related to Glutamic Acid Metabolism and Coronary Heart Disease in Individuals With Type 2 Diabetes. *JAMA*. 2013;310(8):821. doi:10.1001/jama.2013.276305

93. Zheng Y, Hu FB, Ruiz-Canela M, et al. Metabolites of Glutamate Metabolism Are Associated With Incident Cardiovascular Events in the PREDIMED PREvención con DIeta MEDiterránea (PREDIMED) Trial. *J Am Heart Assoc*. 2016;5(9). doi:10.1161/JAHA.116.003755

94. Roy A, Guatimosim S, Prado VF, Gros R, Prado MAM. Cholinergic activity as a new target in diseases of the heart. *Mol Med*. 2014. doi:10.2119/molmed.2014.00125

95. Malin SG, Shavva VS, Tarnawski L, Olofsson PS. Functions of acetylcholine-producing lymphocytes in immunobiology. *Curr Opin Neurobiol*. 2020. doi:10.1016/j.conb.2020.01.017

96. Inanaga K, Ichiki T, Miyazaki R, et al. Acetylcholinesterase inhibitors attenuate atherogenesis in apolipoprotein E-knockout mice. *Atherosclerosis*. 2010;213(1):52-58. doi:10.1016/j.atherosclerosis.2010.07.027

97. Johansson ME, Ulleryd MA, Bernardi A, et al. α7 Nicotinic Acetylcholine Receptor Is Expressed in Human Atherosclerosis and Inhibits Disease in Mice—Brief Report. *Arterioscler Thromb Vasc Biol*. 2014;34(12):2632-2636. doi:10.1161/ATVBAHA.114.303892

98. Sheng Y, Zhu L. The crosstalk between autonomic nervous system and blood vessels. *Int J Physiol Pathophysiol Pharmacol*. 2018;10(1):17-28. http://www.ncbi.nlm.nih.gov/pubmed/29593847.

99. Hashimoto T, Ichiki T, Watanabe A, et al. Stimulation of α7 nicotinic acetylcholine receptor by AR-R17779 suppresses atherosclerosis and aortic aneurysm formation in apolipoprotein E-deficient mice. *Vascul Pharmacol*. 2014;61(2-3):49-55.

doi:10.1016/j.vph.2014.03.006

100. Grill WM, Kirsch RF. Neuroprosthetic Applications of Electrical Stimulation. *Assist Technol*. 2000;12(1):6-20. doi:10.1080/10400435.2000.10132006

101. Larson CE, Meng E. A review for the peripheral nerve interface designer. *J Neurosci Methods*. 2020;332(June 2019):108523. doi:10.1016/j.jneumeth.2019.108523

102. Johnson RL, Wilson CG. A review of vagus nerve stimulation as a therapeutic intervention. *J Inflamm Res*. 2018;Volume 11:203-213. doi:10.2147/JIR.S163248

103. Bonaz B, Bazin T, Pellissier S. The Vagus Nerve at the Interface of the Microbiota-Gut-Brain Axis. *Front Neurosci*. 2018;12. doi:10.3389/fnins.2018.00049

104. D'Haens G, Cabrijan Z, Eberhardson M, et al. P574 The effects of vagus nerve stimulation in biologic-refractory Crohn's disease: A prospective clinical trial. *J Crohn's Colitis*. 2018;12:S397-S398. doi:10.1093/ecco-jcc/jjx180.701

105. Bonaz B, Sinniger V, Pellissier S. Vagus nerve stimulation: a new promising therapeutic tool in inflammatory bowel disease. *J Intern Med*. 2017;282(1):46-63. doi:10.1111/joim.12611

106. NCT01118455. Trial to Assess Vagus Nerve Stimulation Therapy vs. Anti-Epileptic Drug (AED) Treatment in Children With Refractory Seizures. *https://clinicaltrials.gov/show/NCT01118455*. 2010.

107. Neuromodulation to Regulate Inflammation and Autonomic Imbalance in Sepsis. *Case Med Res*. June 2019. doi:10.31525/ct1-nct03992378

108. D'Haens G, Cabrijan Z, Eberhardson M, et al. P574 The effects of vagus nerve stimulation in biologic-refractory Crohn's disease: A prospective clinical trial. *J Crohn's Colitis*. 2018;12(supplement_1):S397-S398. doi:10.1093/ecco-jcc/jjx180.701

109. Koopman FA, Chavan SS, Miljko S, et al. Vagus nerve stimulation inhibits cytokine production and attenuates disease severity in rheumatoid arthritis. *Proc Natl Acad Sci*. 2016;113(29):8284-8289. doi:10.1073/pnas.1605635113

110. Meneses G, Bautista M, Florentino A, et al. Electric stimulation of the vagus nerve reduced mouse neuroinflammation induced by lipopolysaccharide. *J Inflamm*. 2016;13(1):33. doi:10.1186/s12950-016-0140-5

111. 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*. 2017;(121). doi:10.3791/54890

112. Tarnawski L, Reardon C, Caravaca AS, et al. Adenylyl Cyclase 6 Mediates Inhibition of TNF in the Inflammatory Reflex. *Front Immunol*. 2018;9:2648. doi:10.3389/fimmu.2018.02648

113. Noller CM, Levine YA, Urakov TM, Aronson JP, Nash MS. Vagus Nerve Stimulation in Rodent Models: An Overview of Technical Considerations. *Front Neurosci*. 2019;13. doi:10.3389/fnins.2019.00911

114. Downs ME, Lee SA, Yang G, Kim S, Wang Q, Konofagou EE. Non-invasive

peripheral nerve stimulation via focused ultrasound in vivo. *Phys Med Biol*. 2018;63(3):35011. doi:10.1088/1361-6560/aa9fc2

115. Krasovitski B, Frenkel V, Shoham S, Kimmel E. Intramembrane cavitation as a unifying mechanism for ultrasound-induced bioeffects. *Proc Natl Acad Sci U S A*. 2011;108(8):3258-3263. doi:10.1073/pnas.1015771108

116. Zhu L, Altman MB, Laszlo A, et al. Ultrasound Hyperthermia Technology for Radiosensitization. *Ultrasound Med Biol*. 2019;45(5):1025-1043. doi:10.1016/j.ultrasmedbio.2018.12.007

117. Sarvazyan AP, Rudenko O V., Nyborg WL. Biomedical applications of radiation force of ultrasound: Historical roots and physical basis. *Ultrasound Med Biol*. 2010;36(9):1379-1394. doi:10.1016/j.ultrasmedbio.2010.05.015

118. Jakešová M, Silverå Ejneby M, Đerek V, et al. Optoelectronic control of single cells using organic photocapacitors. *Sci Adv*. 2019;5(4):eaav5265. doi:10.1126/sciadv.aav5265

119. Silverå-Ejneby M, Jakešová M, Ferrero J, et al. A chronic photocapacitor implant for noninvasive neurostimulation with deep red light. *bioRxiv*. 2020:2020.07.01.182113. doi:10.1101/2020.07.01.182113

120. A. Sluka K, Smith HS, Walsh DM. Transcutaneous Electrical Nerve Stimulation (TENS). In: *Neuromodulation*. Vol 1. Volume 1. Elsevier; 2009:335-344. doi:10.1016/B978-0-12-374248-3.00025-2

121. Travers MJ, O'Connell NE, Tugwell P, Eccleston C, Gibson W. Transcutaneous electrical nerve stimulation (TENS) for chronic pain: the opportunity to begin again. *Cochrane Database Syst Rev*. 2020;4:ED000139. doi:10.1002/14651858.ED000139

122. Grossman N, Bono D, Dedic N, et al. Noninvasive Deep Brain Stimulation via Temporally Interfering Electric Fields. *Cell*. 2017;169(6):1029-1041.e16. doi:10.1016/j.cell.2017.05.024

123. Mirzakhalili E, Barra B, Capogrosso M, Lempka SF. Biophysics of Temporal Interference Stimulation. *Cell Syst*. 2020;11(6):557-572.e5. doi:10.1016/j.cels.2020.10.004

124. Sheardown M, Nielsen E, Hansen A, Jacobsen P, Honore T. 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. *Science (80-)*. 1990;247(4942):571-574. doi:10.1126/science.2154034

125. Perisic Matic L, Rykaczewska U, Razuvaev A, et al. Phenotypic Modulation of Smooth Muscle Cells in Atherosclerosis Is Associated With Downregulation of LMOD1, SYNPO2, PDLIM7, PLN , and SYNM. *Arterioscler Thromb Vasc Biol*. 2016;36(9):1947-1961. doi:10.1161/ATVBAHA.116.307893

126. Alexa, A; Rahnenfuhrer J. topGO: Enrichment Analysis for Gene Ontology. R package version 2.37.0. Rahnenfuhrer.

127. Tenembaum D. KEGGREST: Client-side REST access to KEGG. R package version 1.28.0. 2020;(R package version 1.28.0.).

128. Luo W, Brouwer C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. *Bioinformatics*. 2013:29(14): 1830-1831.

129. Bannenberg GL, Chiang N, Ariel A, et al. Molecular Circuits of Resolution: Formation and Actions of Resolvins and Protectins. *J Immunol*. 2005;174(7):4345-4355. doi:10.4049/jimmunol.174.7.4345

130. Karadimou G, Folkersen L, Berg M, et al. Low TLR7 gene expression in atherosclerotic plaques is associated with major adverse cardio- and cerebrovascular events. *Cardiovasc Res*. 2017;113(1):30-39. doi:10.1093/cvr/cvw231

131. Wirka RC, Wagh D, Paik DT, et al. Atheroprotective roles of smooth muscle cell phenotypic modulation and the TCF21 disease gene as revealed by single-cell analysis. *Nat Med*. 2019;25(8):1280-1289. doi:10.1038/s41591-019-0512-5

132. Tarnawski L, Gallina AL, Kort EJ, et al. Identification and Characterization of Human Activation-Induced ChAT⁺CD4⁺ T Cells. *bioRxiv*. January 2021:2021.04.27.441632. doi:10.1101/2021.04.27.441632

133. Reardon C, Duncan GS, Brüstle A, et al. Lymphocyte-derived ACh regulates local innate but not adaptive immunity. *Proc Natl Acad Sci*. 2013;110(4):1410-1415. doi:10.1073/pnas.1221655110

134. Watanabe M, Kimura A, Akasaka K, Hayashi S. Determination of acetylcholine in human blood. *Biochem Med Metab Biol*. 1986;36(3):355-362. doi:10.1016/0885-4505(86)90147-7

135. Dutta P, Courties G, Wei Y, et al. Myocardial infarction accelerates atherosclerosis. *Nature*. 2012;487(7407):325-329. doi:10.1038/nature11260

136. Chen L, Xin X, Eckhart AD, Yang N, Faber JE. Regulation of Vascular Smooth Muscle Growth by α1-Adrenoreceptor Subtypes in Vitro and in Situ. *J Biol Chem*. 1995;270(52):30980-30988. doi:10.1074/jbc.270.52.30980

137. Gallina AL, Rykaczewska U, Wirka RC, et al. AMPA-Type Glutamate Receptors Associated With Vascular Smooth Muscle Cell Subpopulations in Atherosclerosis and Vascular Injury. *Front Cardiovasc Med*. 2021;8(April):1-17. doi:10.3389/fcvm.2021.655869

138. Perisic L, Aldi S, Sun Y, et al. Gene expression signatures, pathways and networks in carotid atherosclerosis. *J Intern Med*. 2016;279(3):293-308. doi:10.1111/joim.12448

139. Röhl S, Rykaczewska U, Seime T, et al. Transcriptomic profiling of experimental arterial injury reveals new mechanisms and temporal dynamics in vascular healing response. *JVS Vasc Sci*. 2020;1:13-27. doi:10.1016/j.jvssci.2020.01.001

140. Thompson S, Chesher D. Lot-to-Lot Variation. *Clin Biochem Rev*. 2018;39(2):51-60. http://www.ncbi.nlm.nih.gov/pubmed/30473592.

141. Caravaca AS, Gallina AL, Tarnawski L, et al. An Effective Method for Acute Vagus Nerve Stimulation in Experimental Inflammation. *Front Neurosci*. 2019;13. doi:10.3389/fnins.2019.00877

142. Harnack D, Winter C, Meissner W, Reum T, Kupsch A, Morgenstern R. The effects of

electrode material, charge density and stimulation duration on the safety of high-frequency stimulation of the subthalamic nucleus in rats. *J Neurosci Methods*. 2004;138(1-2):207-216. doi:10.1016/J.JNEUMETH.2004.04.019

143. Dalli J, Colas RA, Arnardottir H, Serhan CN. Vagal Regulation of Group 3 Innate Lymphoid Cells and the Immunoresolvent PCTR1 Controls Infection Resolution. *Immunity*. 2017;46(1):92-105. doi:10.1016/j.immuni.2016.12.009